Notice Dates

Original notifier submission, dated March 25, 2016……FDA / CVM 00001

First notifier revision, dated October 31, 2016……FDA / CVM 01719

Second notifier revision, dated December 12, 2016……FDA / CVM 01849

Modified *Saccharomyces cerevisiae*
DSM 2194-001

THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED

By
Bio-based Products & Services
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Delft, The Netherlands

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Parsippany, NJ

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1. General introduction and claim of exemption from premarket approval requirements

1.1 Executive Summary

The purpose of this document is to provide technical and scientific information that supports the Generally Recognized as Safe (GRAS) status of DSM's Inactivated Modified *Saccharomyces cerevisiae* (also marketed as Advanced *Saccharomyces cerevisiae*) as a nutritional product for use in poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets, based on scientific procedures.

DSM Bio-based Products and Services (BPS) developed the yeast; our sister company, DSM Nutritional Products will manufacture and package the product. DSM-BPS will market the product. Inactivated Modified *Saccharomyces cerevisiae* is used by bio-ethanol facilities for the fermentation of sugars released from plant-based feed stocks, such as corn, wheat and sorghum into ethanol in a manner similar to the production of beer or grain neutral spirits used for in food and pharmaceutical processing. The yeast is not meant for fermentation of cellulosic feedstock.

Although the yeast was developed for use in bio-ethanol production from food grade substrates such as corn, wheat and sorghum, DSM-BPS is aware that potential customers may separate the inactivated yeast from the spent stillage for sale as an ingredient for animal feed or it will be dried with the cellulosic material in the stillage and become part of the Wet or Dried Distillers Grains with or without solubles (DDG/S and DDG) depending upon the business practices of the potential customer. These practices are commonly followed today with conventional *Saccharomyces cerevisiae* and the use of the yeast and DDG/S are permitted under feed ingredient definitions of the American Association of Feed Control Officers (AAFCO) as overseen by FDA's Center for Veterinary Medicine, at levels not exceeding good manufacturing procedures (AAFCO O.P. Sections 96 and 27 respectively). DSM-BPS does not intend to exceed the current limits for use of yeast and merely seeks to expand its permitted use to a genetically engineered strain.

The subject yeast, used for bio-ethanol production, will be produced in two product forms: a concentrated 'cream yeast' and a dry form commonly referred to as 'active dry yeast'. It is anticipated that the dry form will be the predominant marketed form for bio-ethanol production. Initially the Modified *Saccharomyces cerevisiae* is produced by (b)(4) in a facility that follows food cGMP (21 CFR 110). The yeast is then used in bio-ethanol production where it multiplies several times during the fermentation cycle. Since the host organism is safe and the incorporated DNA does not encode any known harmful or toxic substances, the resulting genetically engineered organism is considered to be safe for use in bio-ethanol production, and the resulting by-products containing the inactivated yeast are also considered safe.

Saccharomyces cerevisiae is a ubiquitous microorganism found all over the globe and has been utilized by humans serendipitously and intentionally for the production of bread, beer and wine for over six thousand years. The National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules (Department of Health and Human Services 1986) considers *Saccharomyces cerevisiae* a safe organism.

The safety of the Inactivated Modified *Saccharomyces cerevisiae* has been established according to the published literature. A comprehensive search of the scientific literature for

safety and toxicity information on *Saccharomyces cerevisiae* was conducted by DSM Bio-based Products and Services. The databases searched included Medline, BIOSIS and EMBASE databases as provided by the host STN.

The compositional equivalence of Modified *Saccharomyces cerevisiae* is shown by the nutritional analysis of two pilot plant batches and a sub-sample of one commercial batch of Modified *Saccharomyces cerevisiae* as compared to the published nutritional data of active dry baker's yeast and to the nutritional analysis of a sample of commercial baker's yeast. As anticipated, because the Modified *Saccharomyces cerevisiae* is the same in basic molecular composition as conventional yeast there was no significant difference between the different yeast products. Additionally, a study conducted to ascertain if excessive amounts of sugar alcohols or organic acids were produced due to the altered metabolism of the yeast did not reveal any differences compared to the wild type parent or a commercially available yeast used for bioethanol manufacture.Therefore we believe the Modified *Saccharomyces cerevisiae* is substantially similar to traditional yeast utilized in animal food.

All relevant information was reviewed, summarized and incorporated into a GRAS dossier, 'THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED' and submitted to the GRAS Panel. The GRAS Panel received information pertaining to the method of manufacture, product specification, analytical data, intended use levels in animal food for avian, ruminant and porcine species, resulting consumption estimates from the intended uses, and any other relevant data on safety and tolerance-related information. The members of the Expert Panel were Mark Cook, PhD, John A. Thomas, PhD, Fellow, ATS, and Stanley M. Tarka, Jr., PhD (Panel Chair).

1.2 Administrative information

1.2.1 Name and address of Notifier

DSM Innovation, Inc.

BioProducts & Services Division

45 Waterview Blvd.

Parsippany, New Jersey, 07054, USA

Tel: 973-257-8500

Person responsible for the dossier:

James La Marta, Ph.D., CFS

45 Waterview Boulevard
Parsippany, New Jersey 07054
Tel: 973-257-8325

1.2.2 Name and Address of Manufacturer

DSM Nutritional Products
1416 Willamsburg County Hwy
Kingstree, NC 29556

1.3 Name and Address of the Distributor

DSM Innovation, Inc.
BioProducts & Services Division
45 Waterview Blvd
Parsippany, NJ 07054

1.4 Common or usual name of the notified substance

The substance that is the subject of this GRAS notification is:

Inactivated Modified *Saccharomyces cerevisiae*

It will be sold alone or as a portion of Distillers Dried Grains with or without Solubles (DDG /DDGS).

1.5 Intended conditions of use and technical effects of the notified substance

The Inactivated Modified *Saccharomyces cerevisiae* will be used as a nutrient source for production animals; poultry; turkeys, breeders, layers and broilers; swine; growers, gilts, sows and piglets; bovines; beef cattle and dairy cows; and minor species such a ducks, quail, sheep and goats. It will be present in dry distillers' grains with or without solubles at up to 20% of the dry solids.

1.6 Basis for GRAS determination

Pursuant to 21 C.F.R. § 570.30(a)(1), a panel of independent experts assembled by DSM, the GRAS Panel, has been asked to review, through scientific procedures, to determine if Inactivated Modified *Saccharomyces cerevisiae* that could be sold as a single feed ingredient or become part of Distillers Dried Grains with or without Solubles and is intended to be used as a

nutrient source, is substantially equivalent to the host strain, common baker's yeast, from which it is derived, and is therefore Generally Recognized As Safe for use as an ingredient in animal food.

1.7 GRAS Exemption Claim

DSM Food Specialties provided the appropriate information on the safety and utility of the notified substance to an independent panel of qualified experts, the GRAS Panel for their evaluation. The enclosed dossier contains the information on the identity of the production organism, manufacture of the commercial product and information supporting the safety of its intended use. Also included are copies of the pertinent literature and other supportive data.

(b)(4) meeting appropriate food-grade specifications as described in this dossier and manufactured consistent with current Good Manufacturing Practices (cGMP), is Generally Recognized As Safe (GRAS) based on scientific procedures and is therefore exempt from the requirement for premarket approval noted in Section 201 (s) of the Federal Food Drug and Cosmetic Act.

1.8 Availability of information for FDA review

The data and information that are the basis for DSM's GRAS determination are available for the FDA's review and copying upon request during normal business hours at:

DSM Innovation, Inc.
BioProducts & Services Division
45 Waterview Blvd
Parsippany, NJ 07054
Tel: 973-257-8500

James La Marta, Ph.D., CFS

Date: 25 March 2016

2. Detailed information about the identity and manufacture of the notified substance CONFIDENTIAL

The subject of this notice is the yeast, Modified *Saccharomyces cerevisiae*' inactivated by the biofuel manufacturing process.

2.1 Description of Modified *Saccharomyces cerevisiae*

Table 2-1 Description of Mofdified *Saccharomyces cerevisiae*

Generic name	*Saccharomyces cerevisiae*
Synonyms	Baker's yeast, Brewer's Yeast
Chemical abstract service number (CAS)	(b)(4)

2.1.1 Taxonomy

Kingdom: Fungi
Phylum: Ascomycota
Class: Saccharomycetes
Order: Saccharomycetales
Family: Saccharomycetaceae
Genus: *Saccharomyces*
Species: *cerevisiae*

The Modified *Saccharomyces cerevisiae* is taxonomically identical to traditional yeast that is used in animal food.

2.2 Modifications introduced to the microorganism

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Nevertheless, the primary products produced by fermentations utilizing the engineered yeast are yeast biomass and ethanol and in that respect our engineered yeast is not different from the conventional yeast normally used for alcoholic fermentations.

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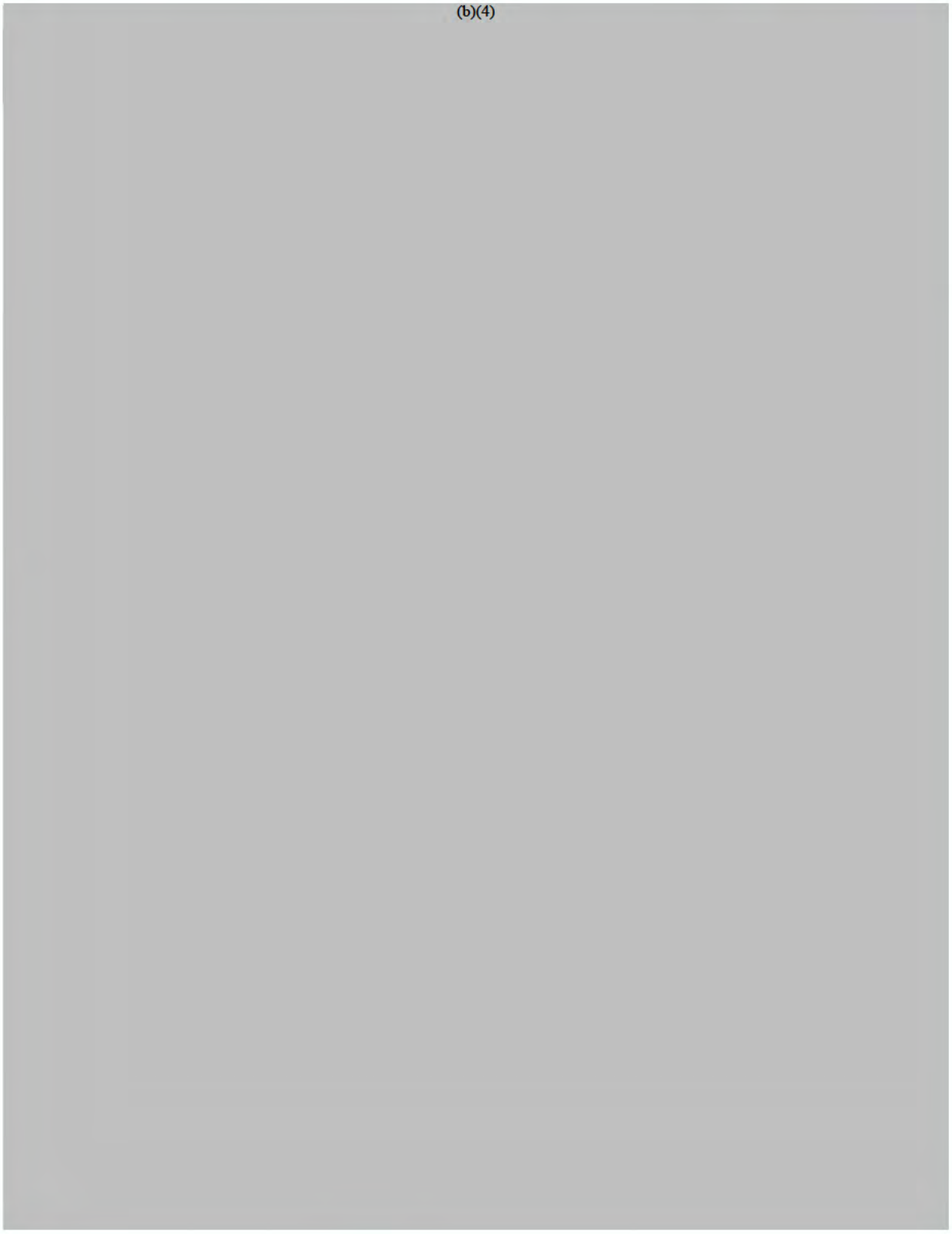

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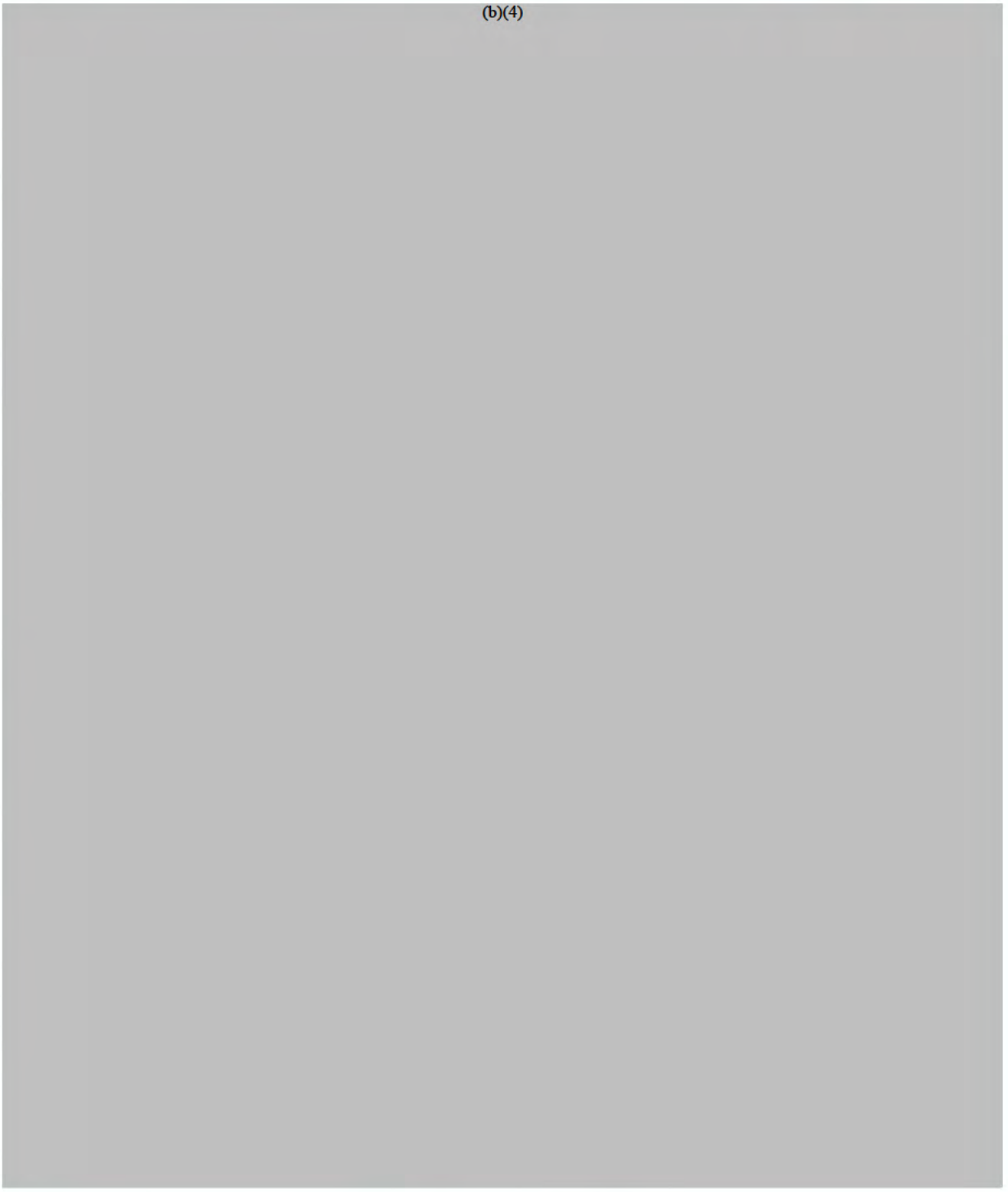

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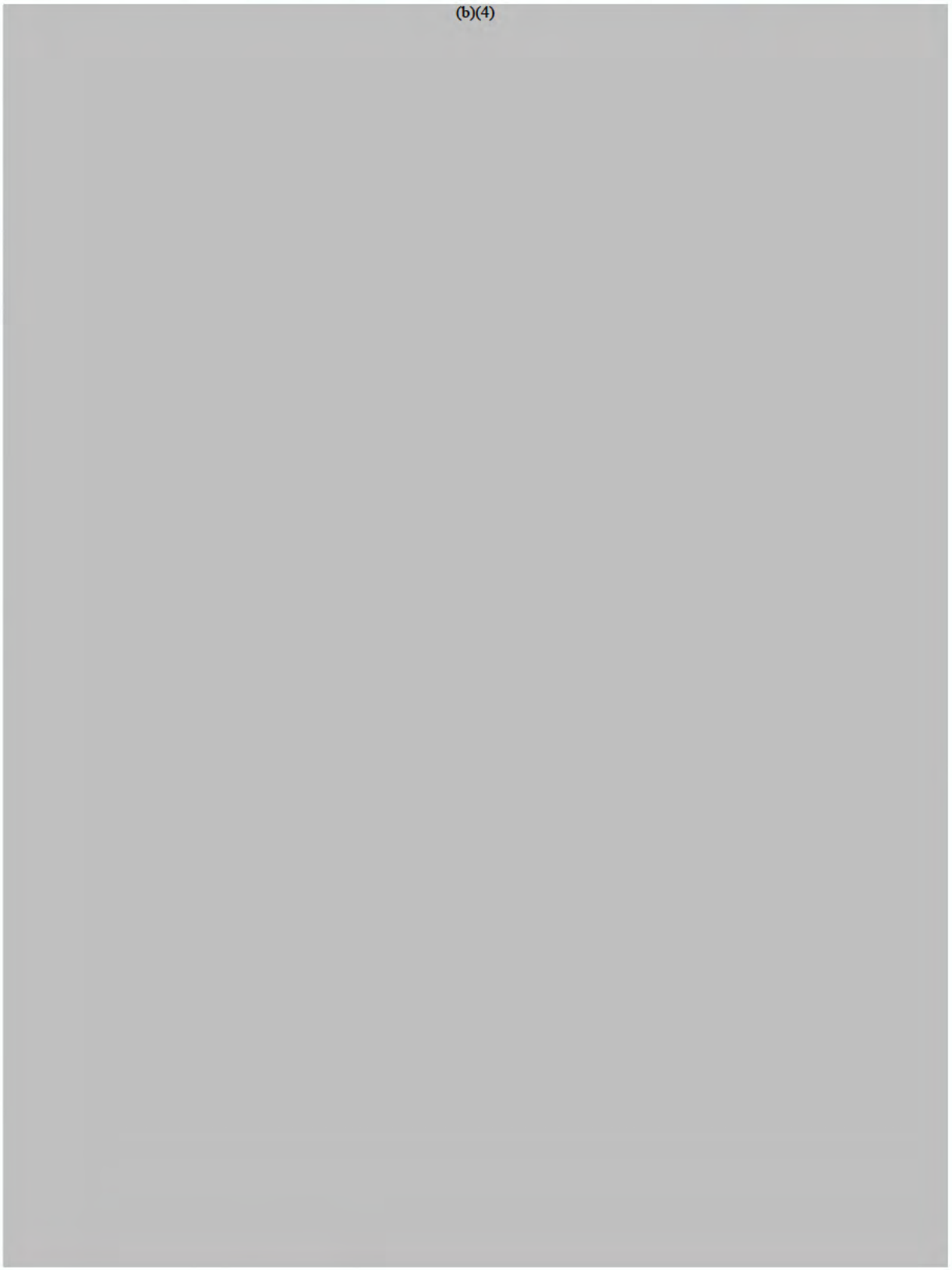

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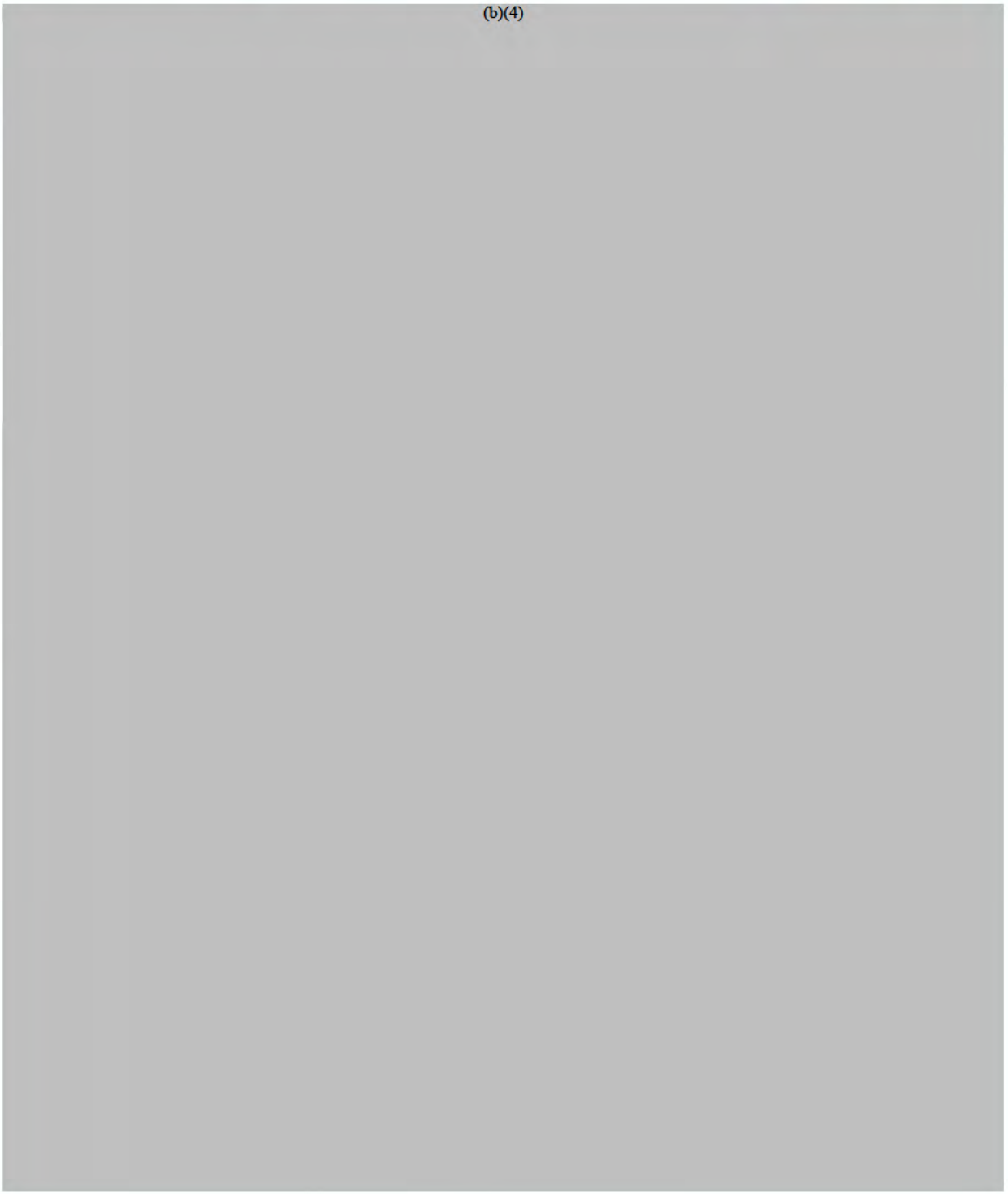

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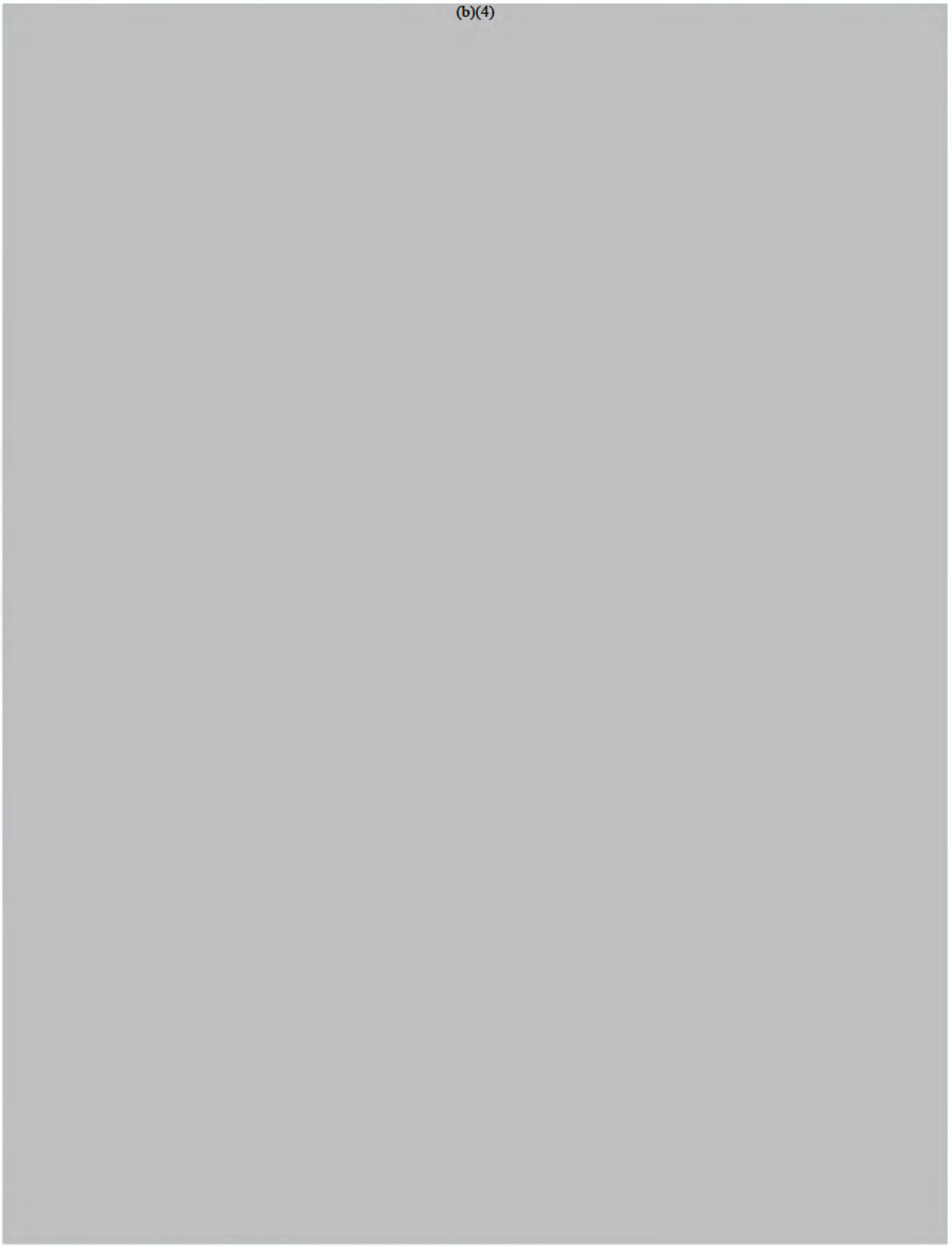

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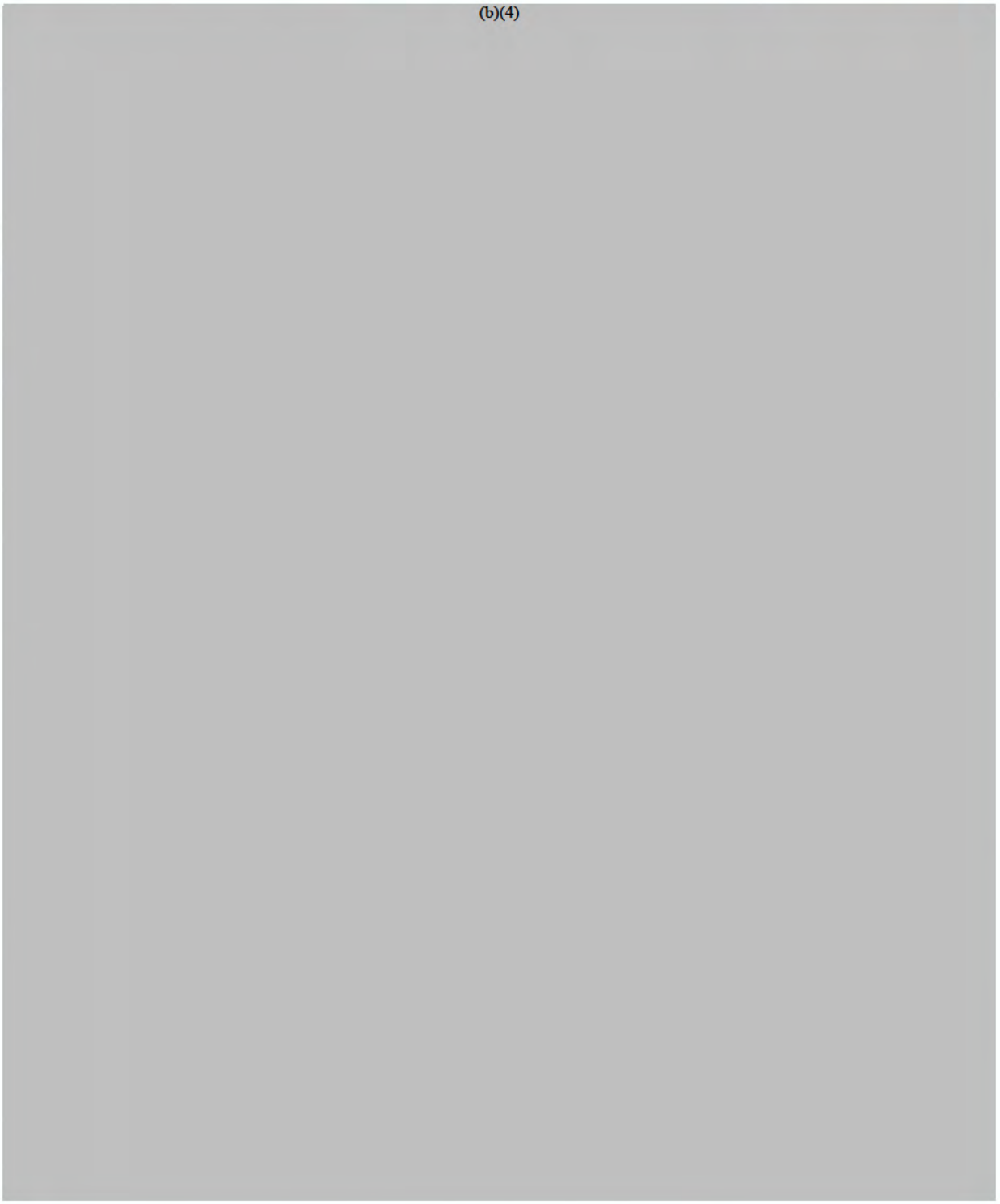

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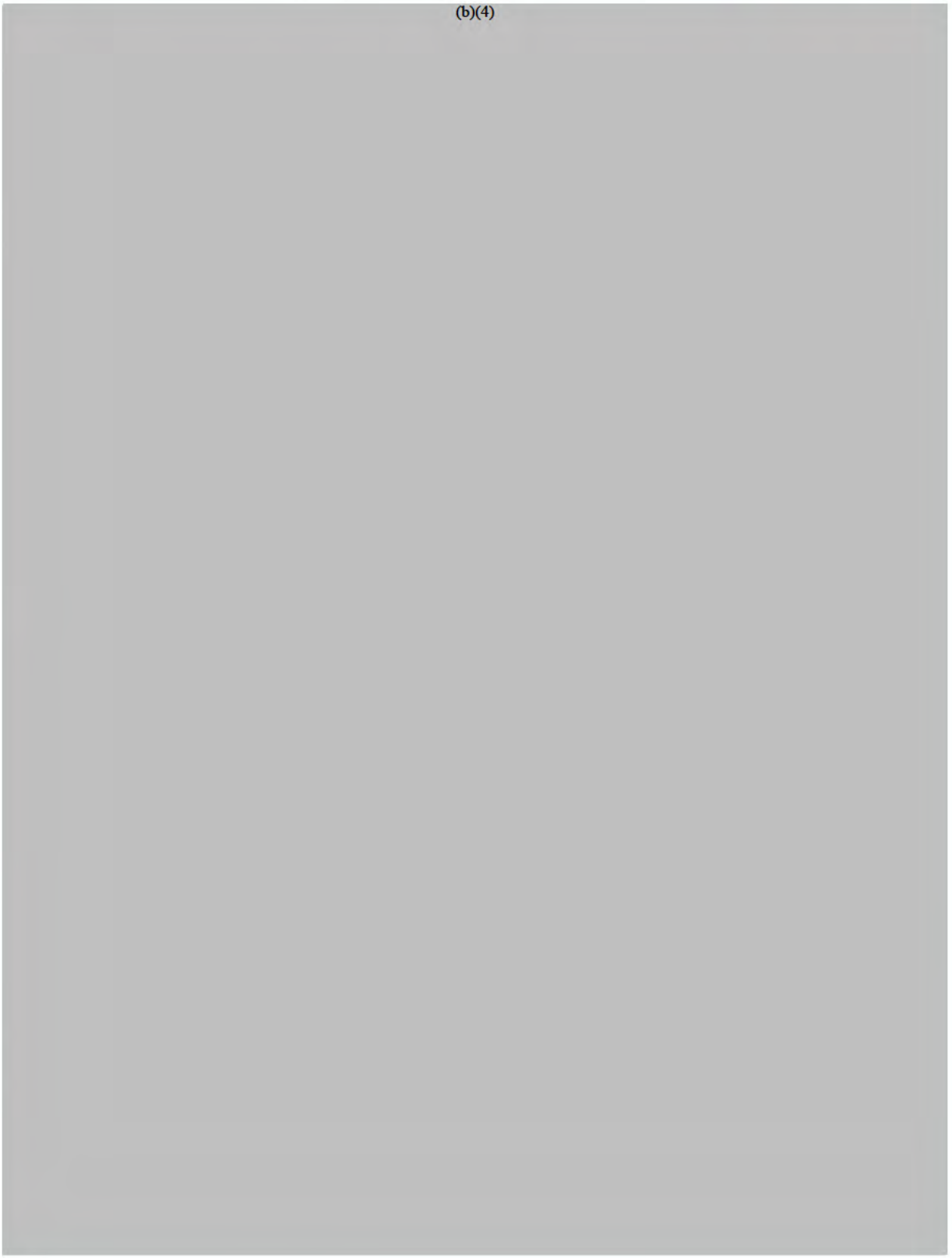

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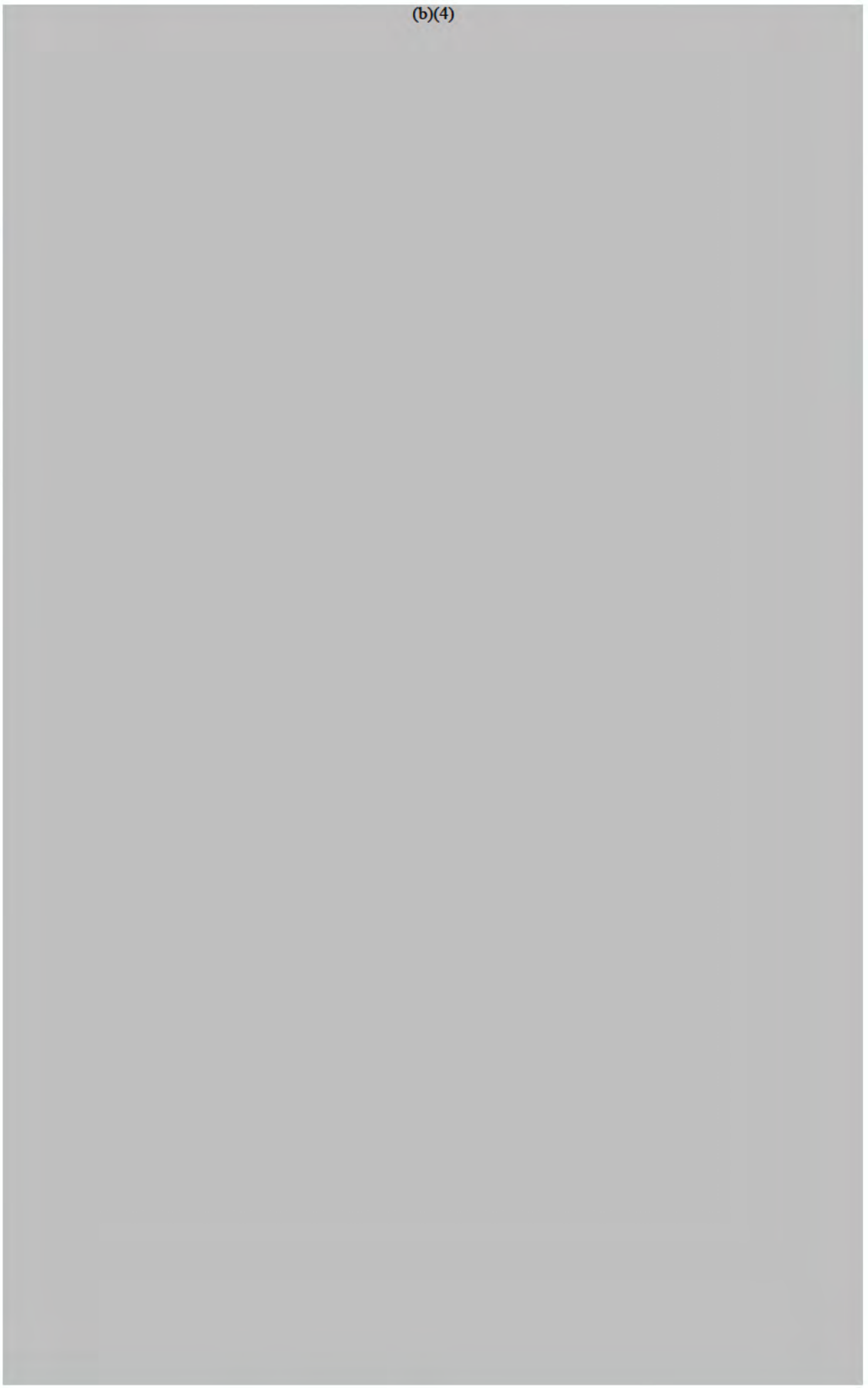

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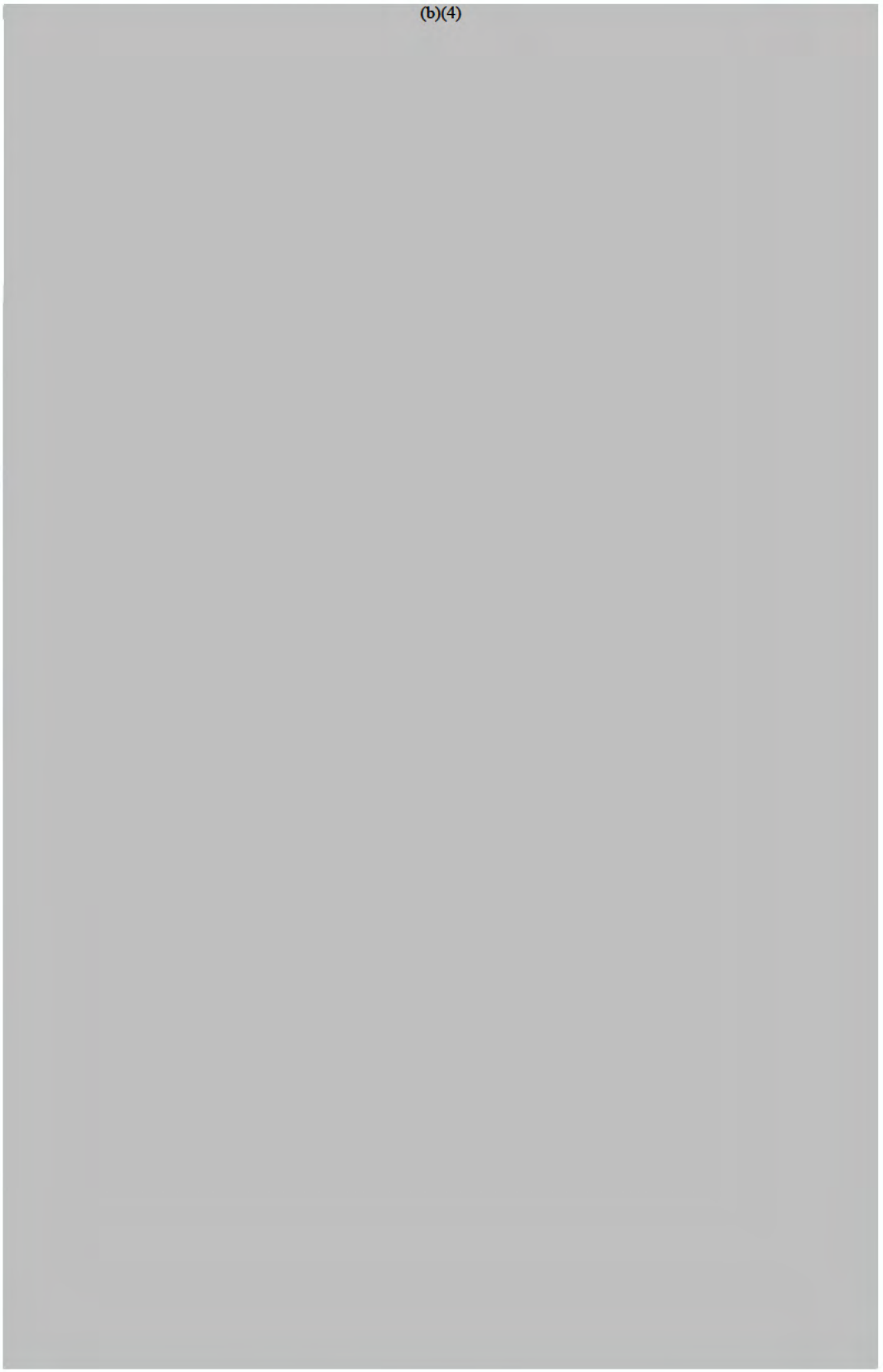

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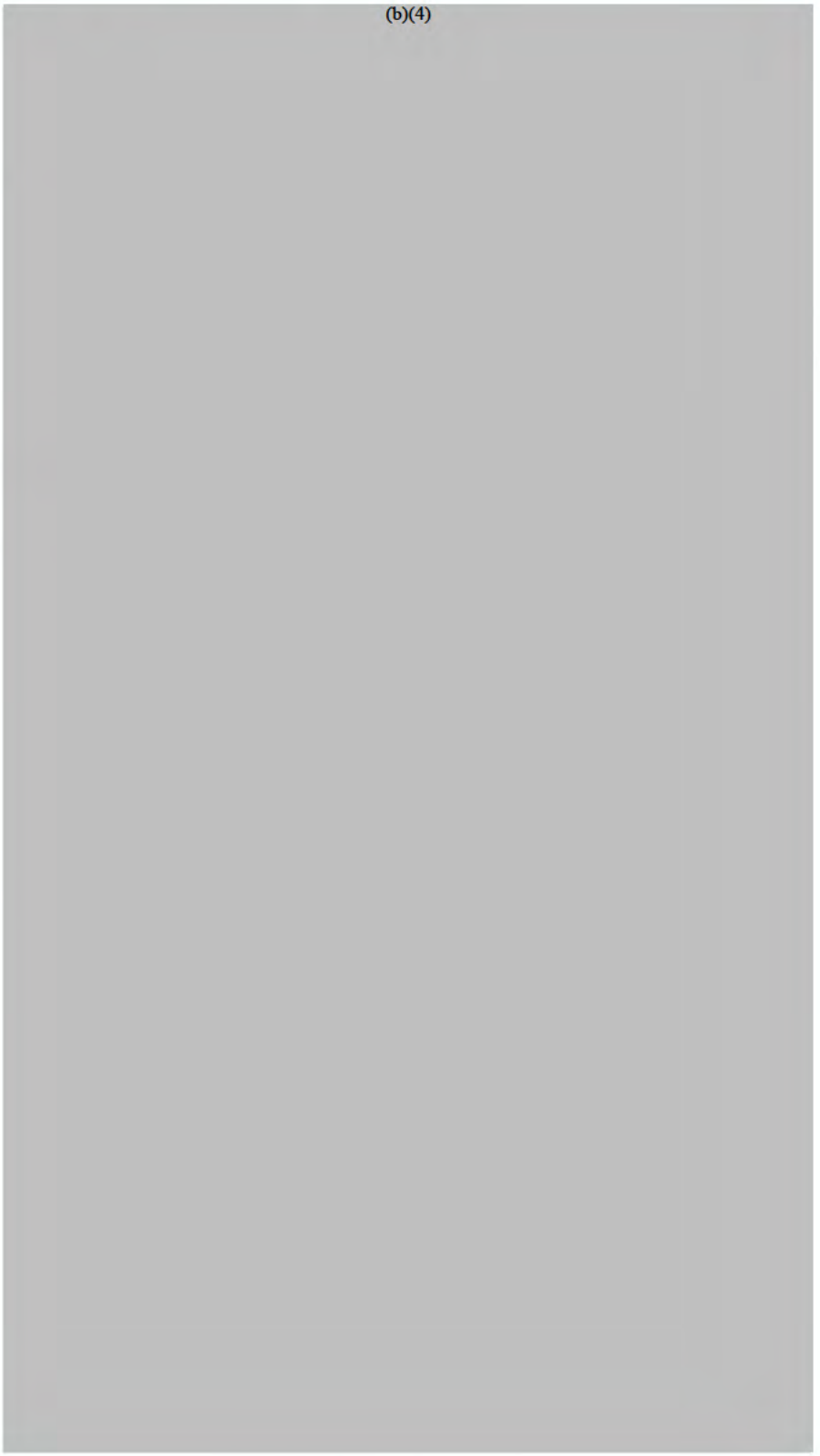

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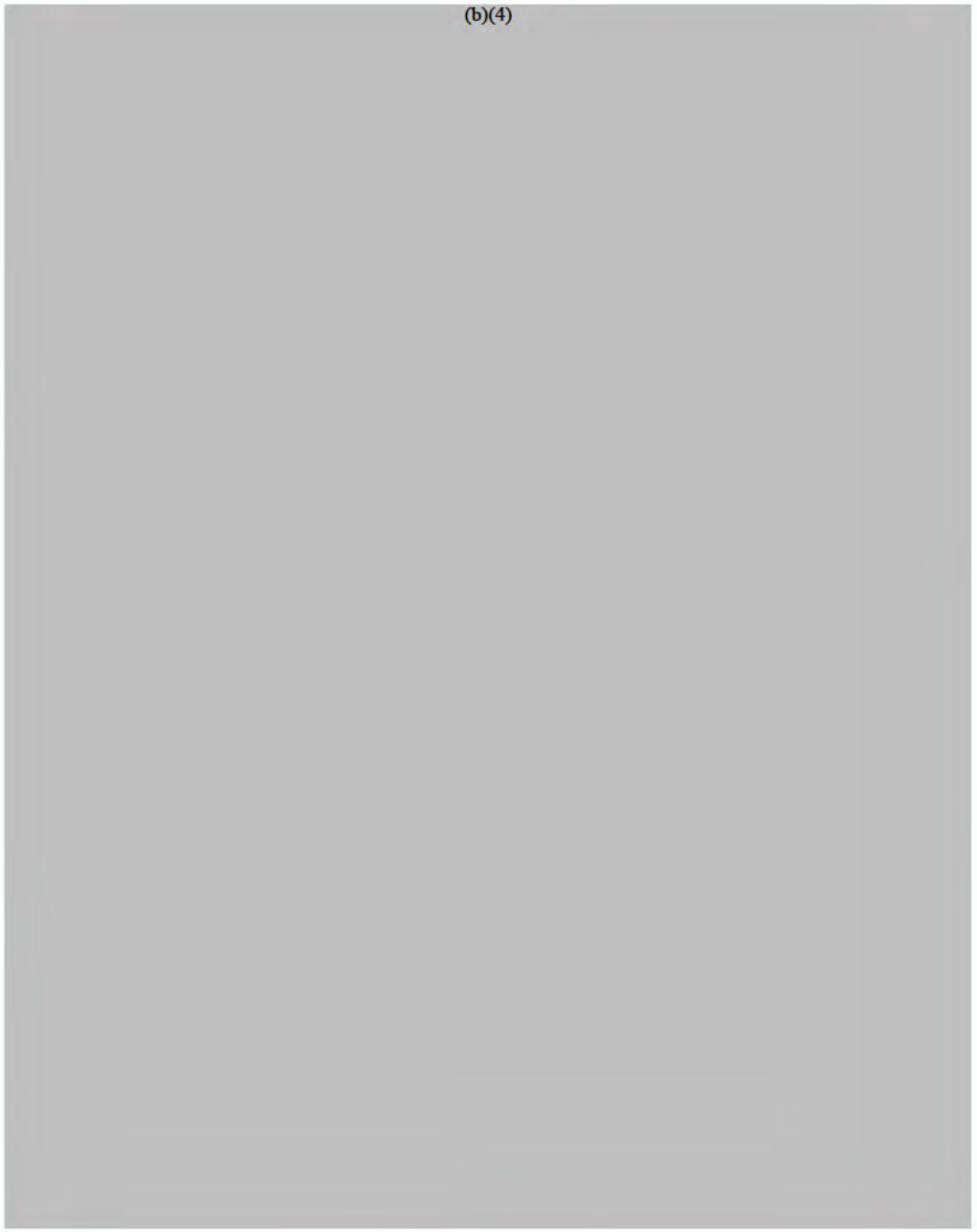

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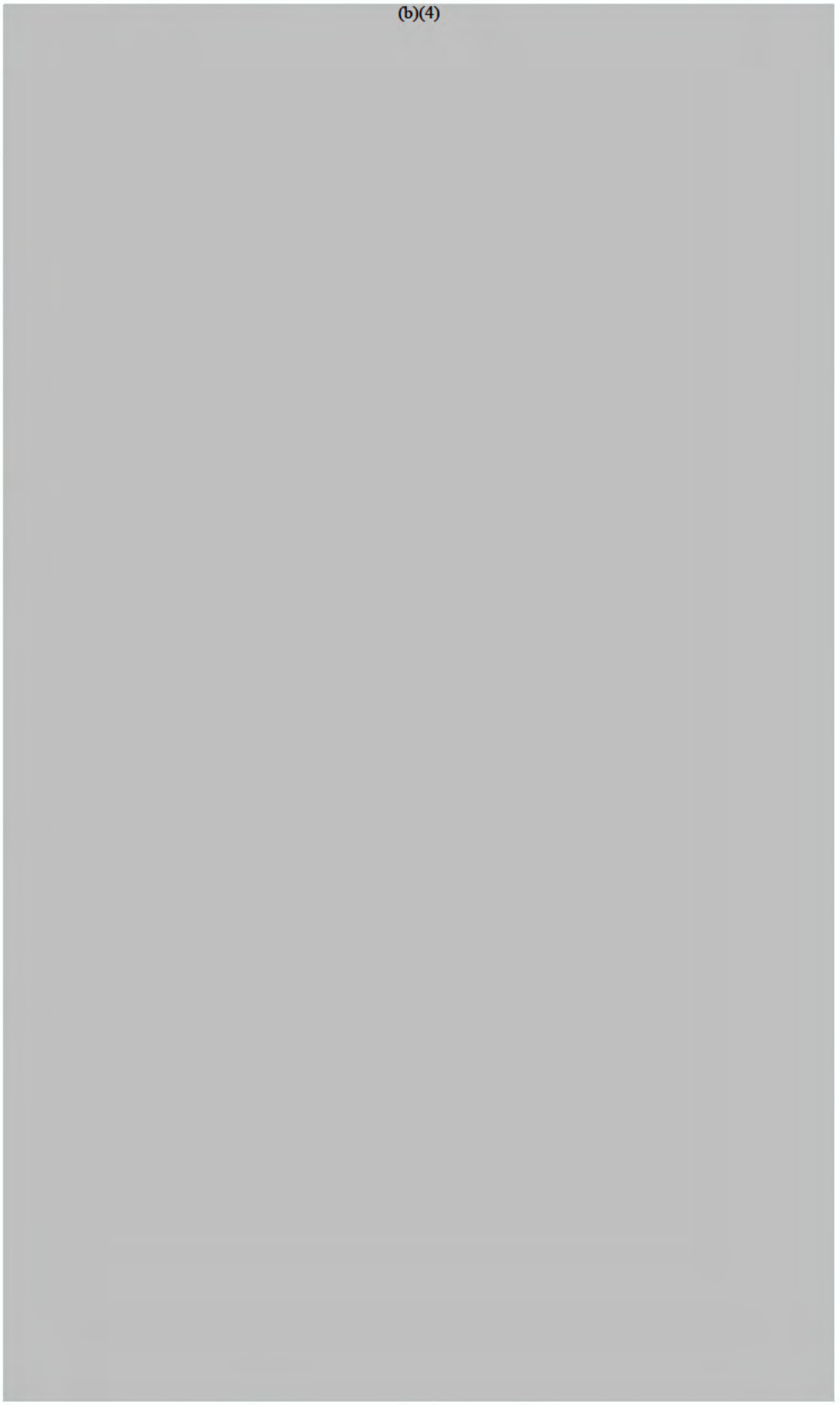

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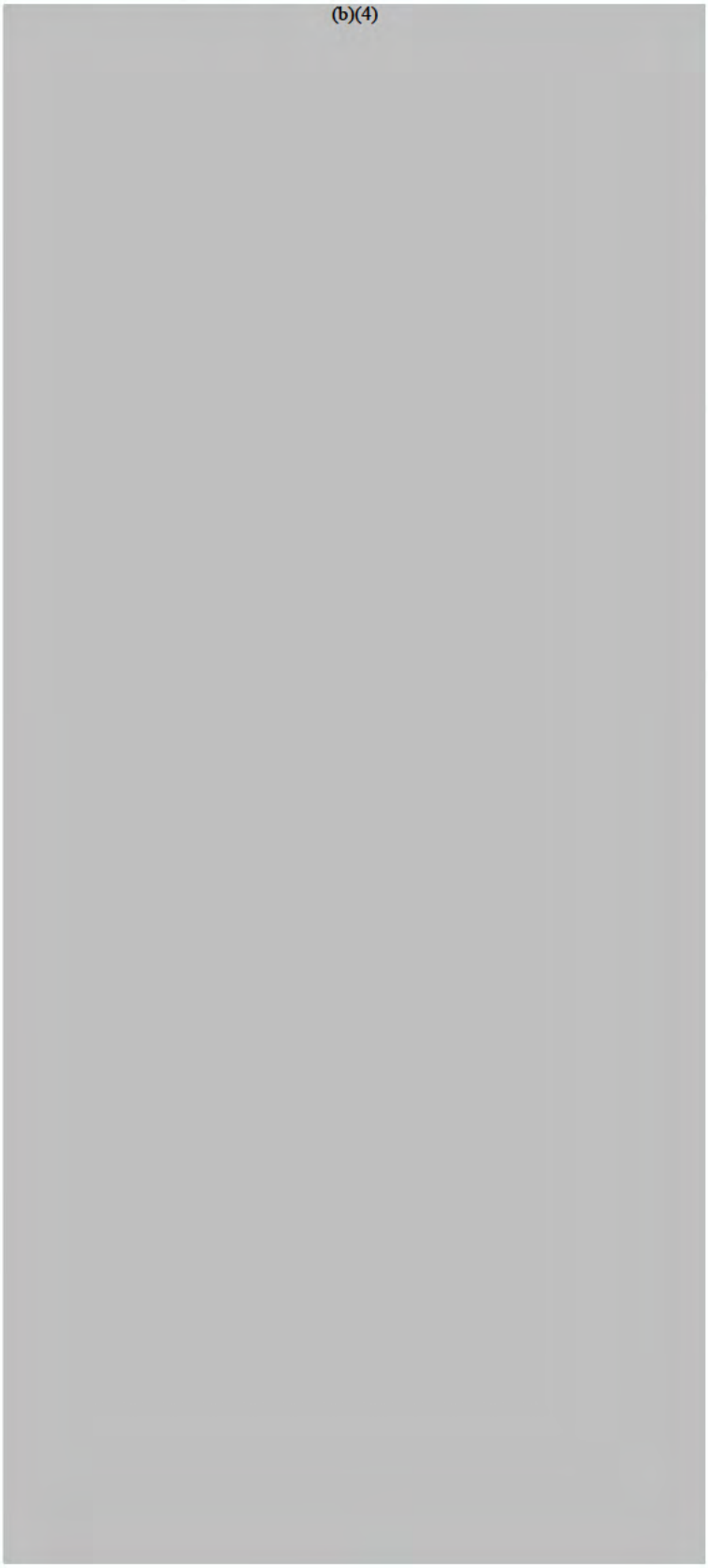

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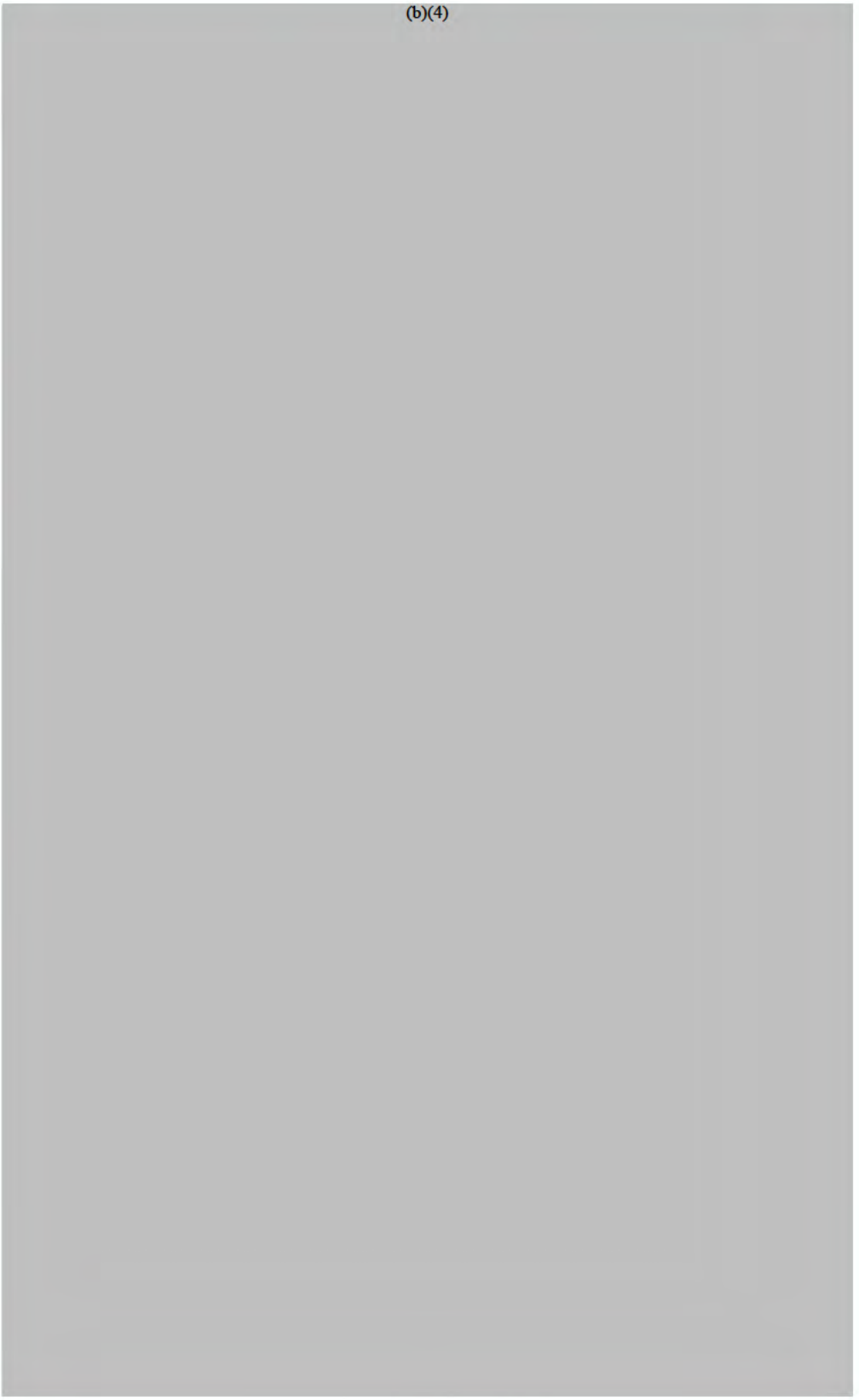

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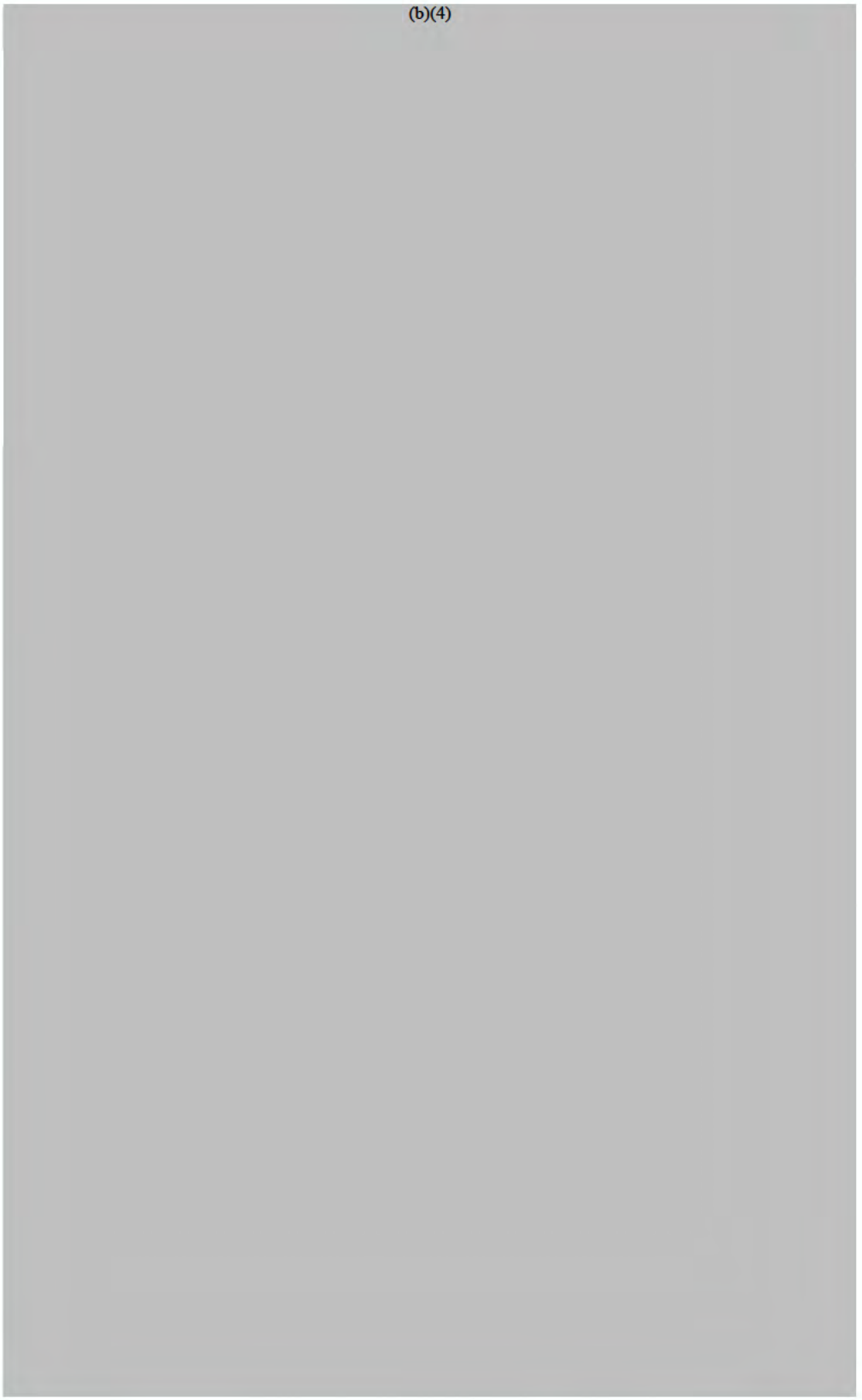

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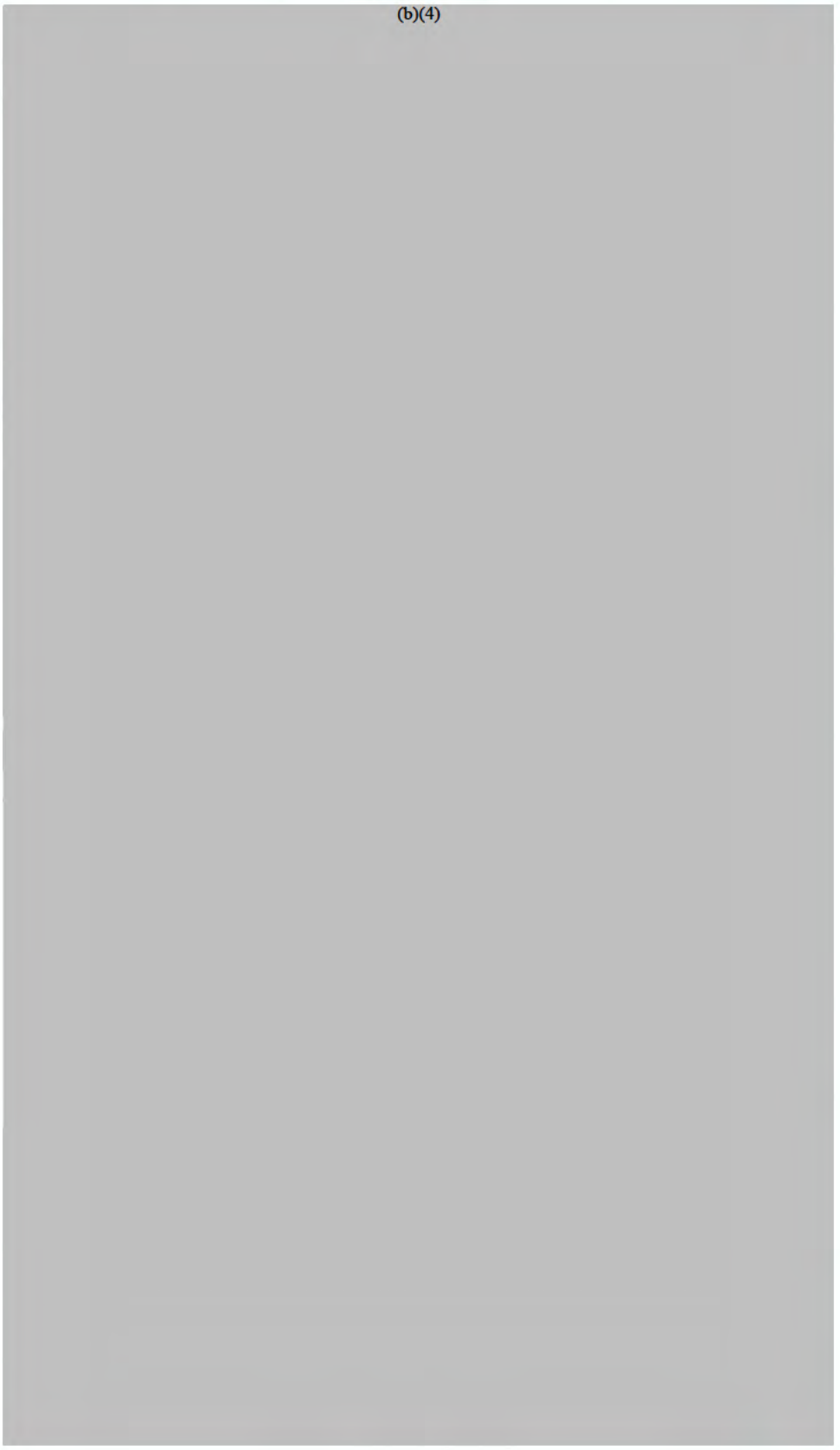

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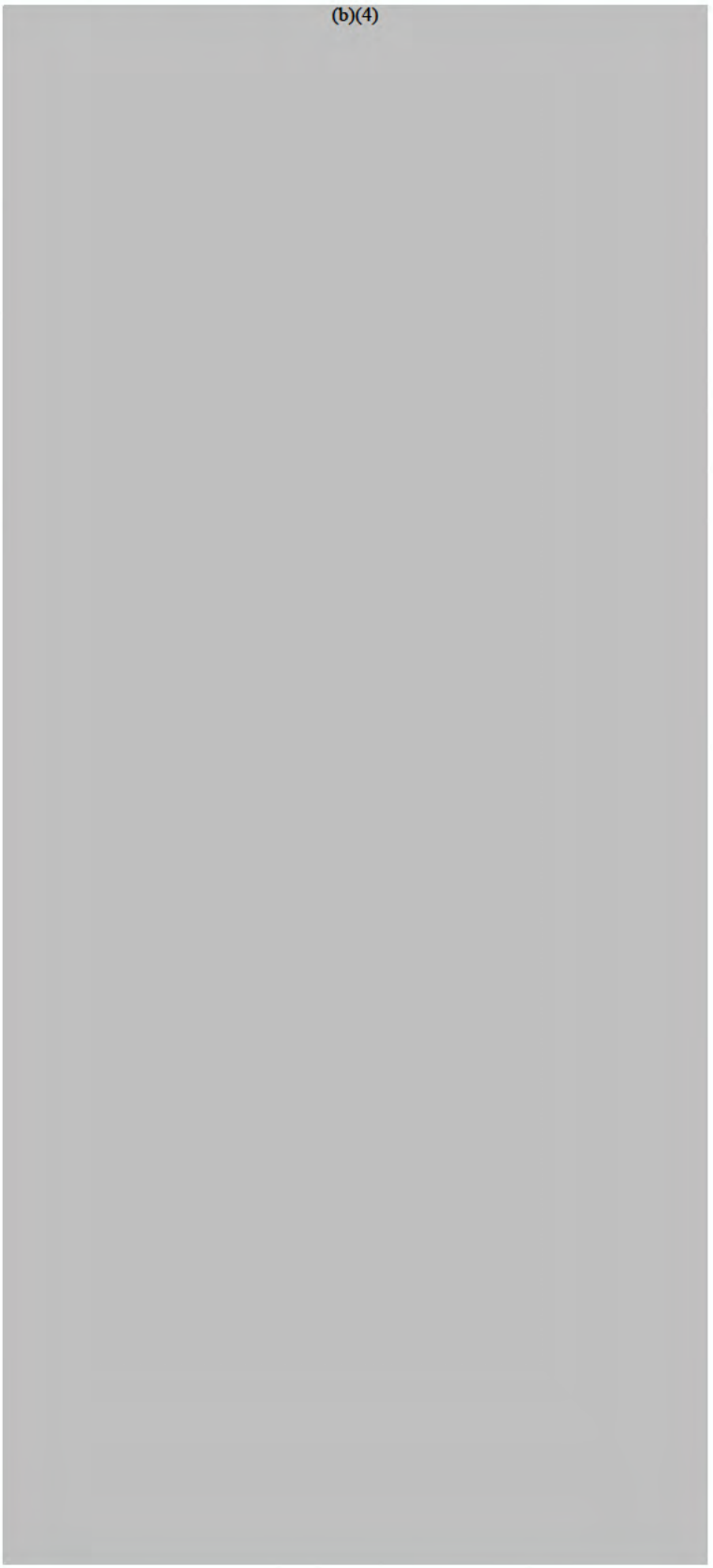

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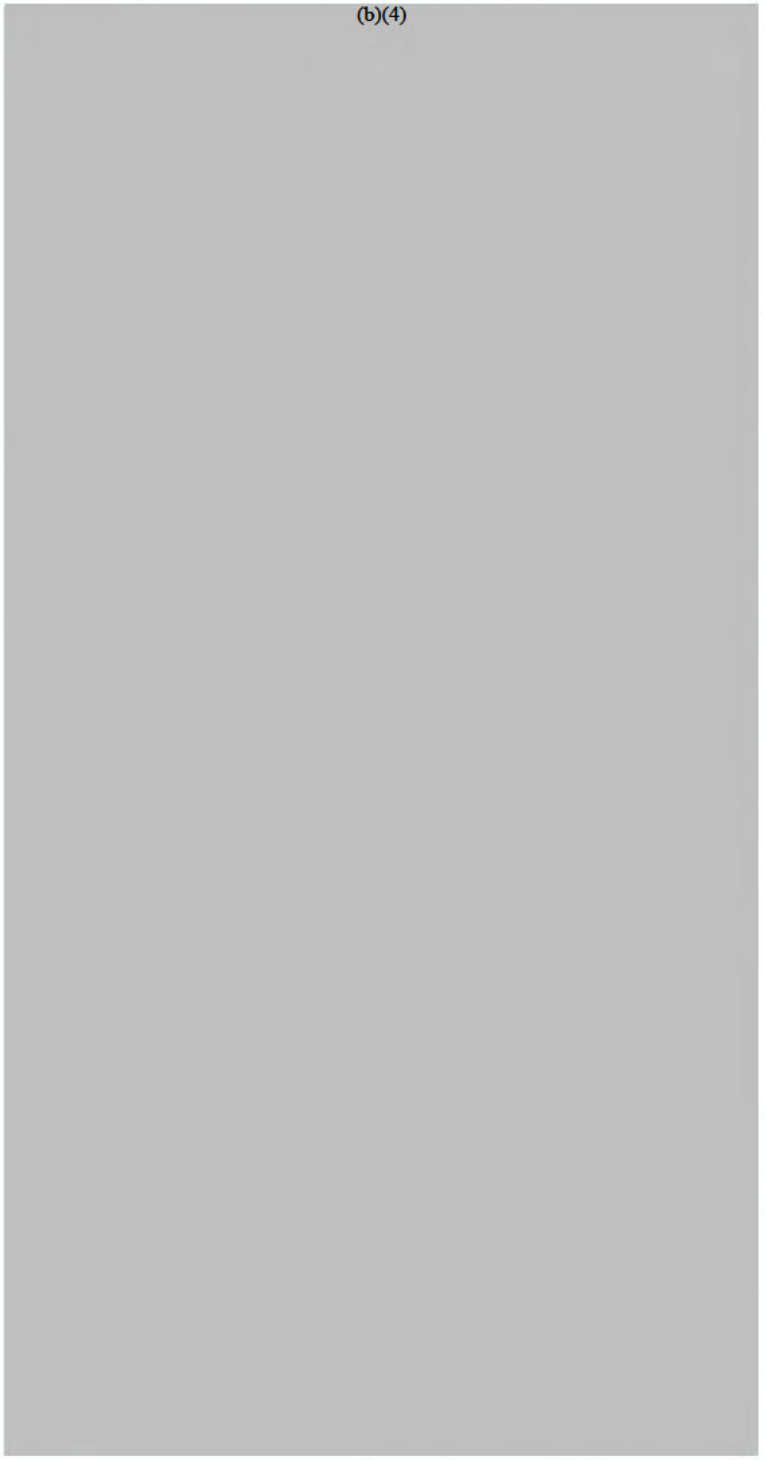

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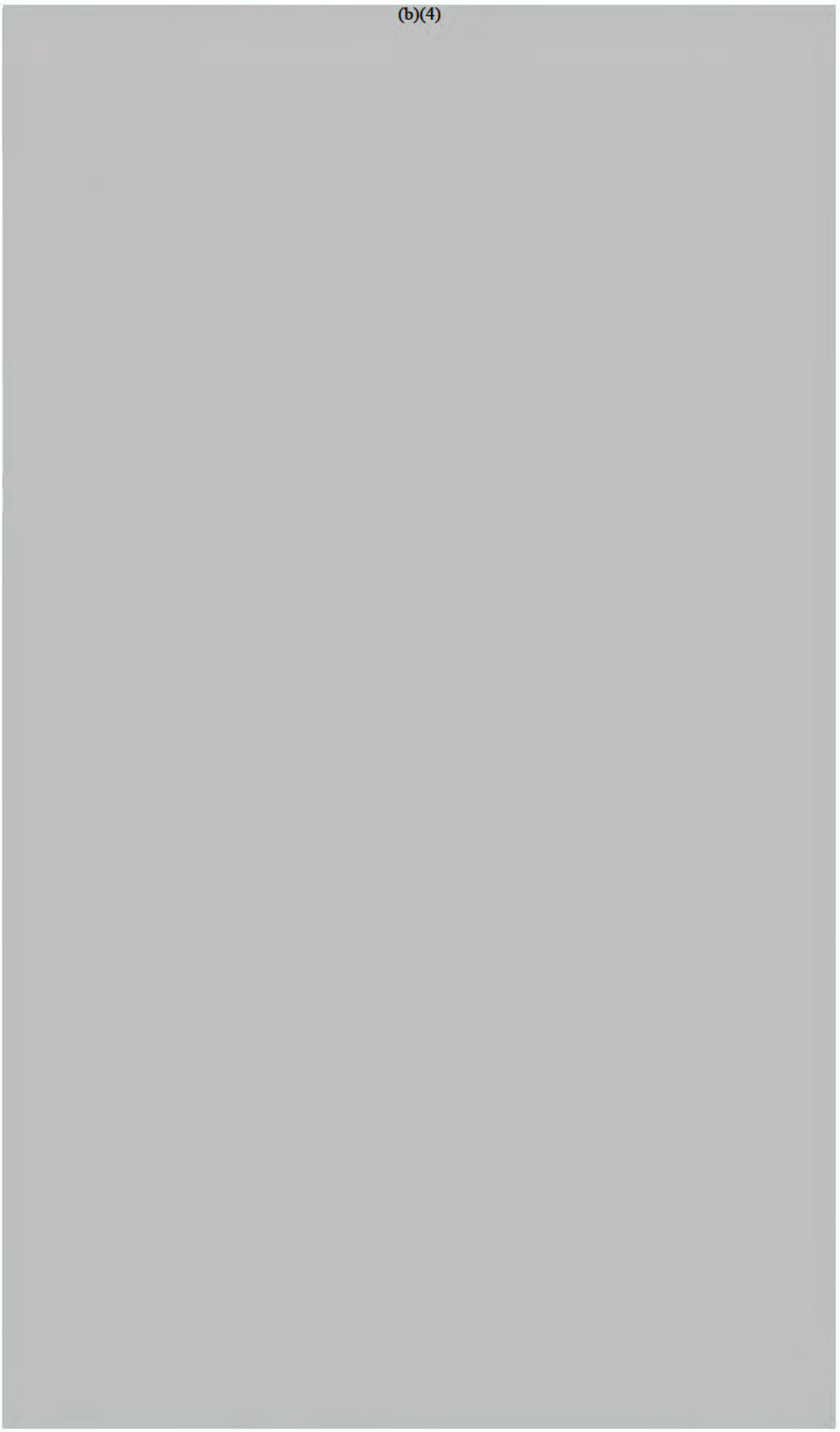

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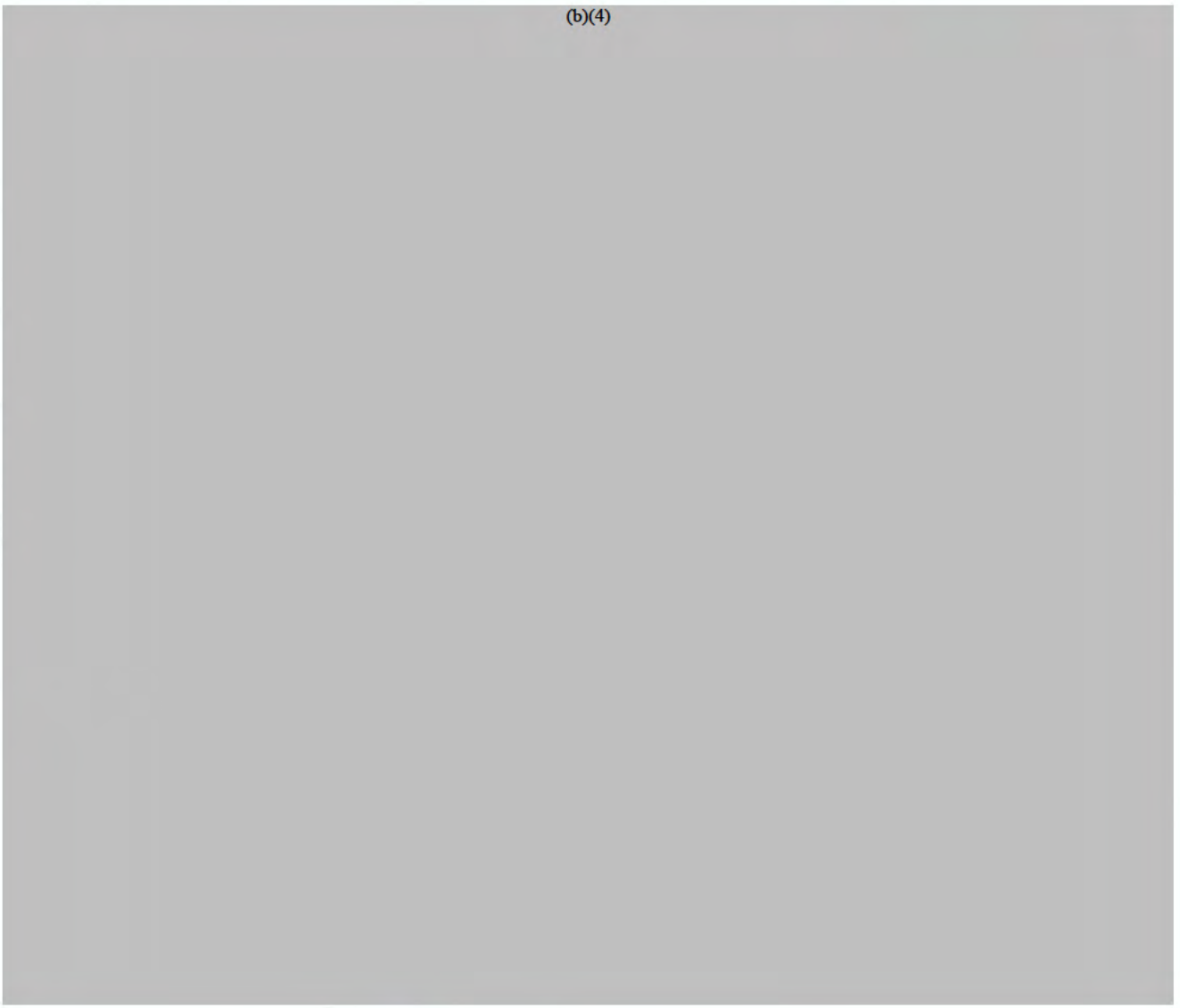

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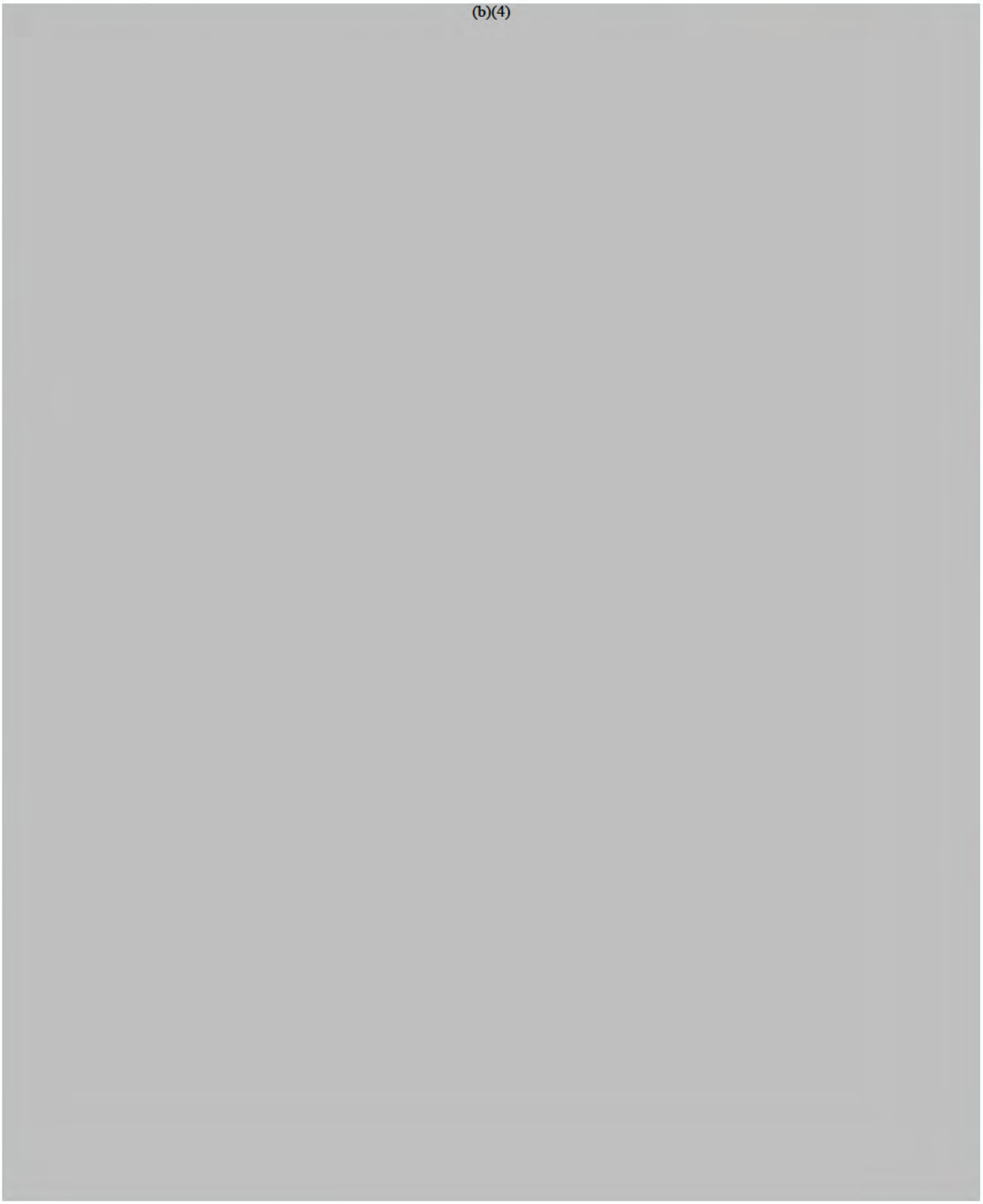

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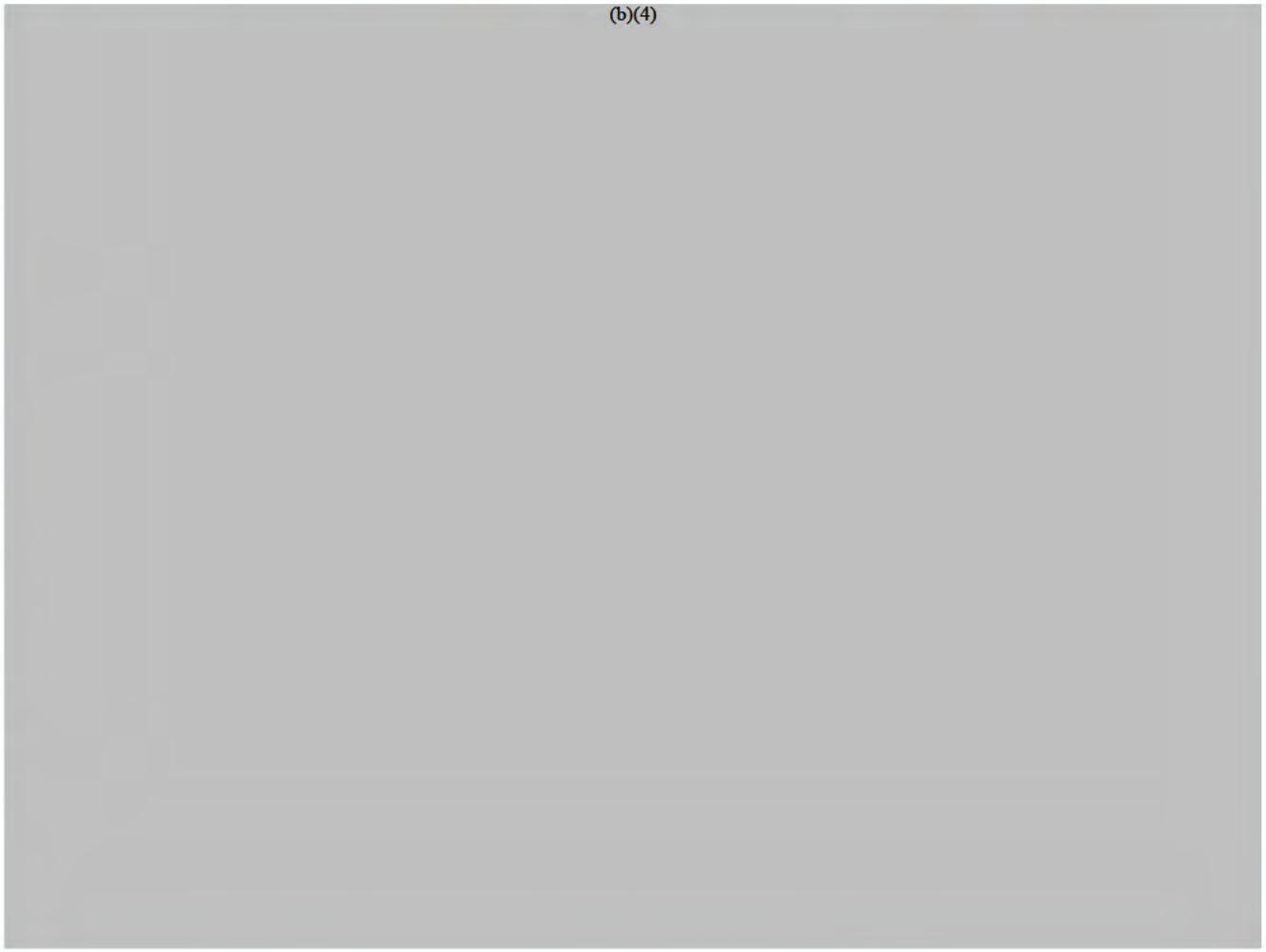

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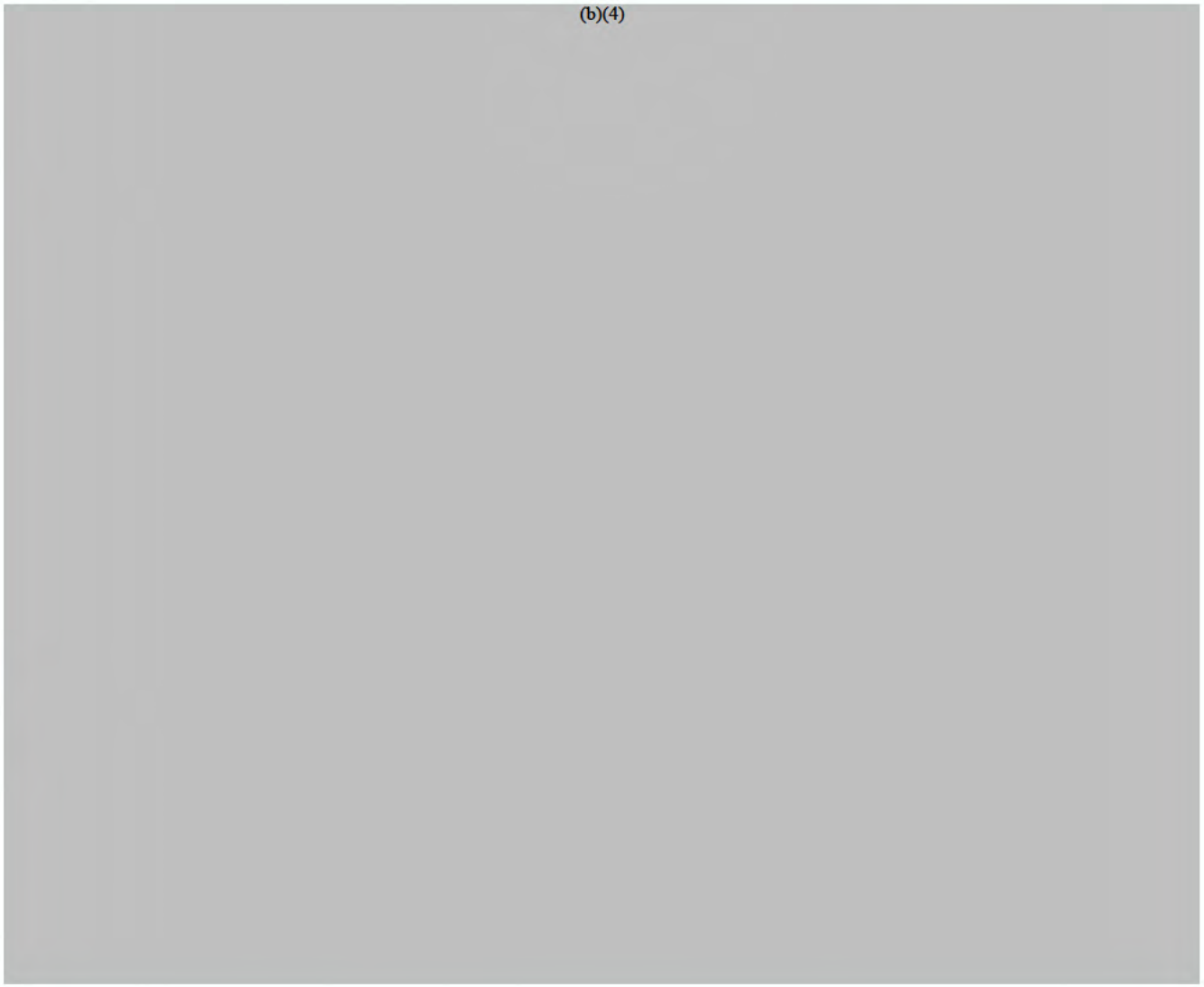

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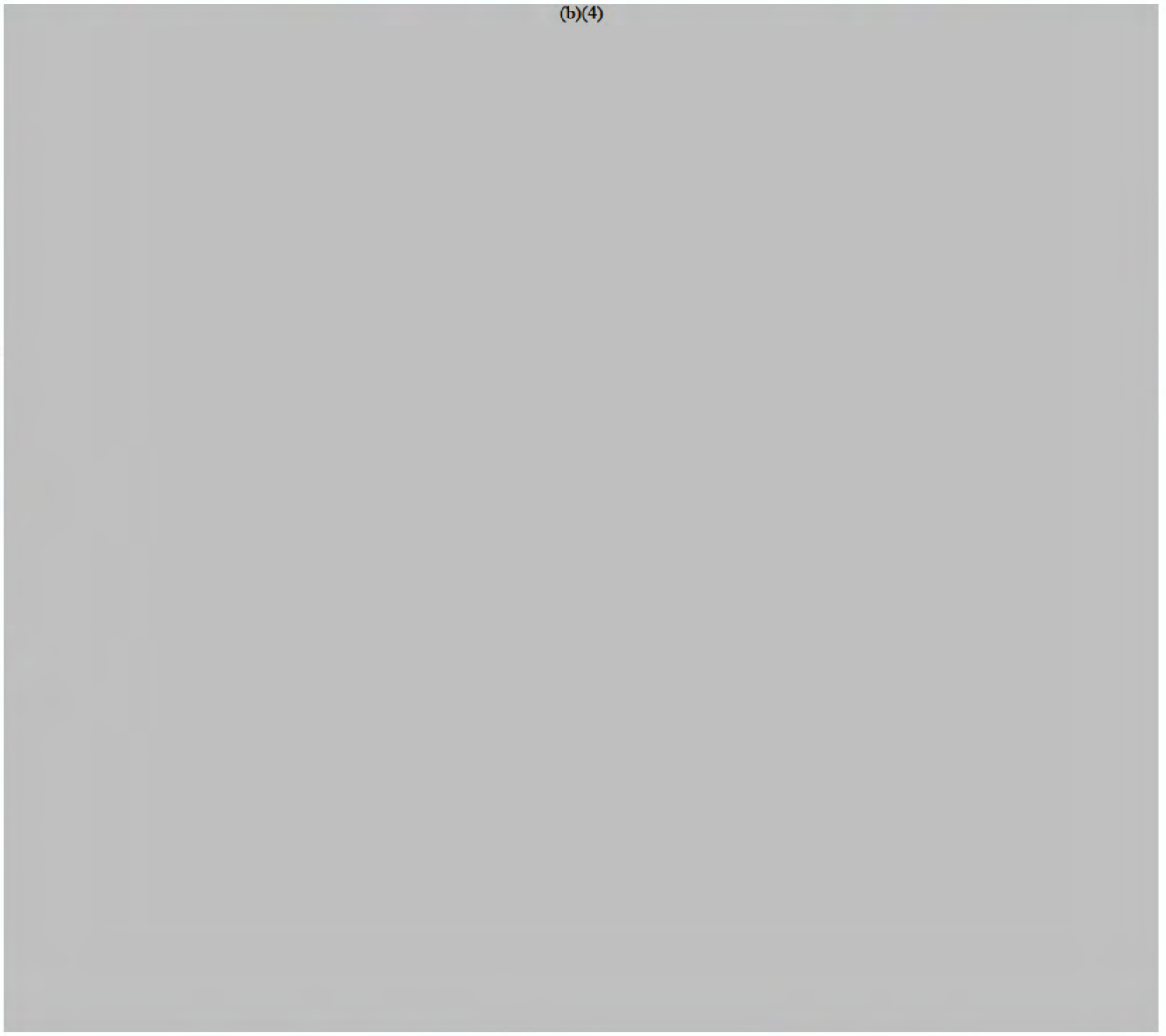

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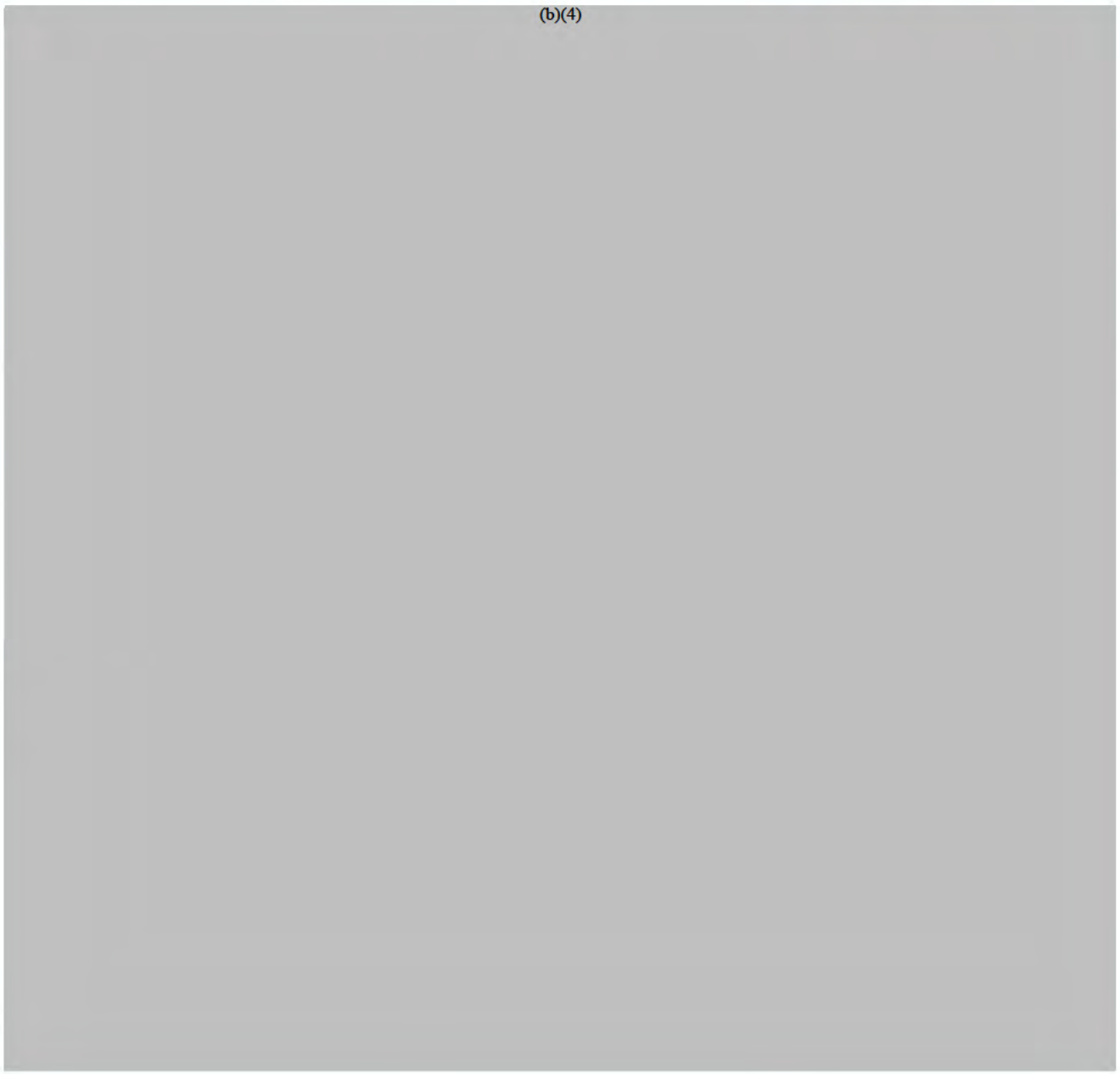

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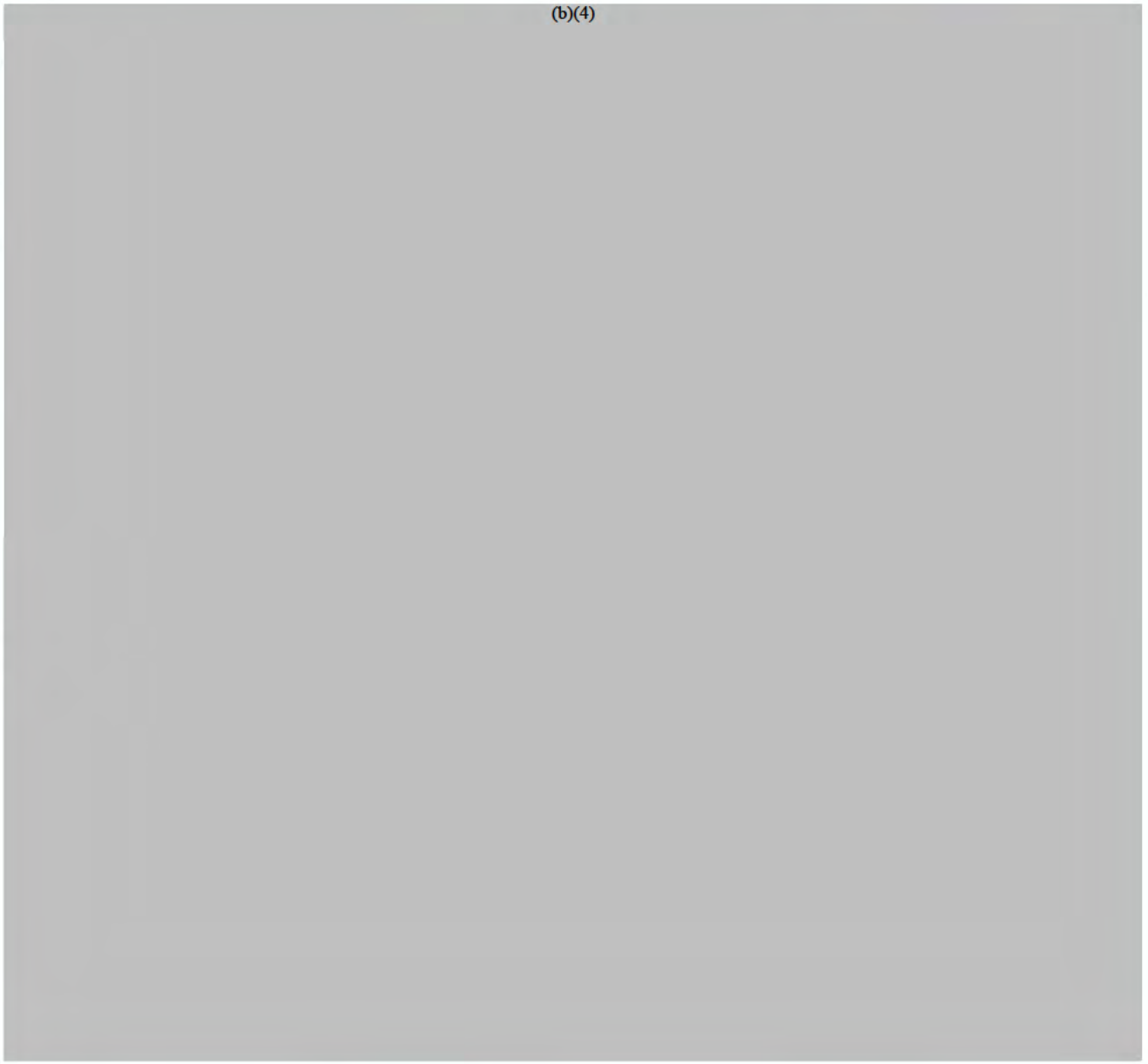

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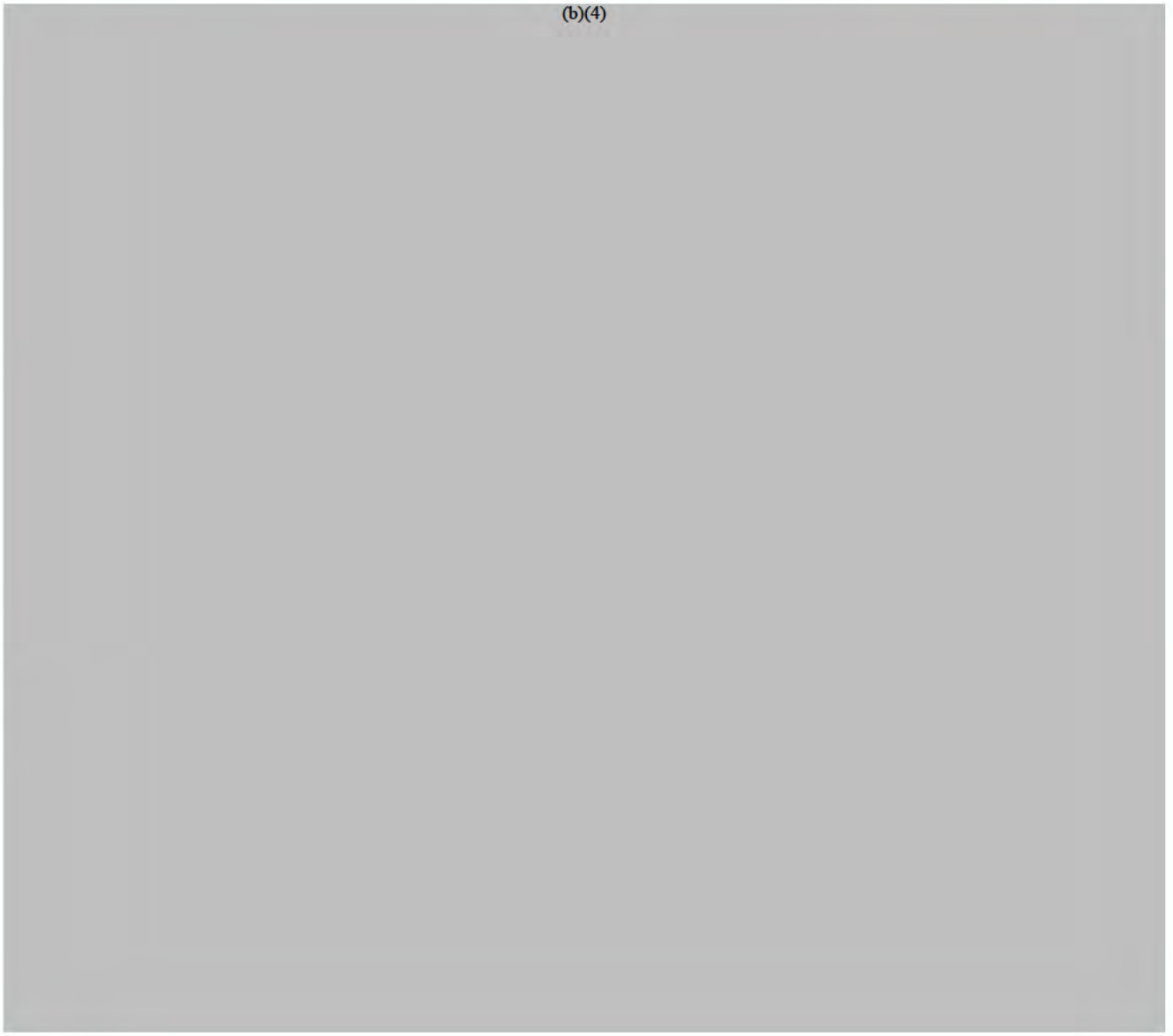

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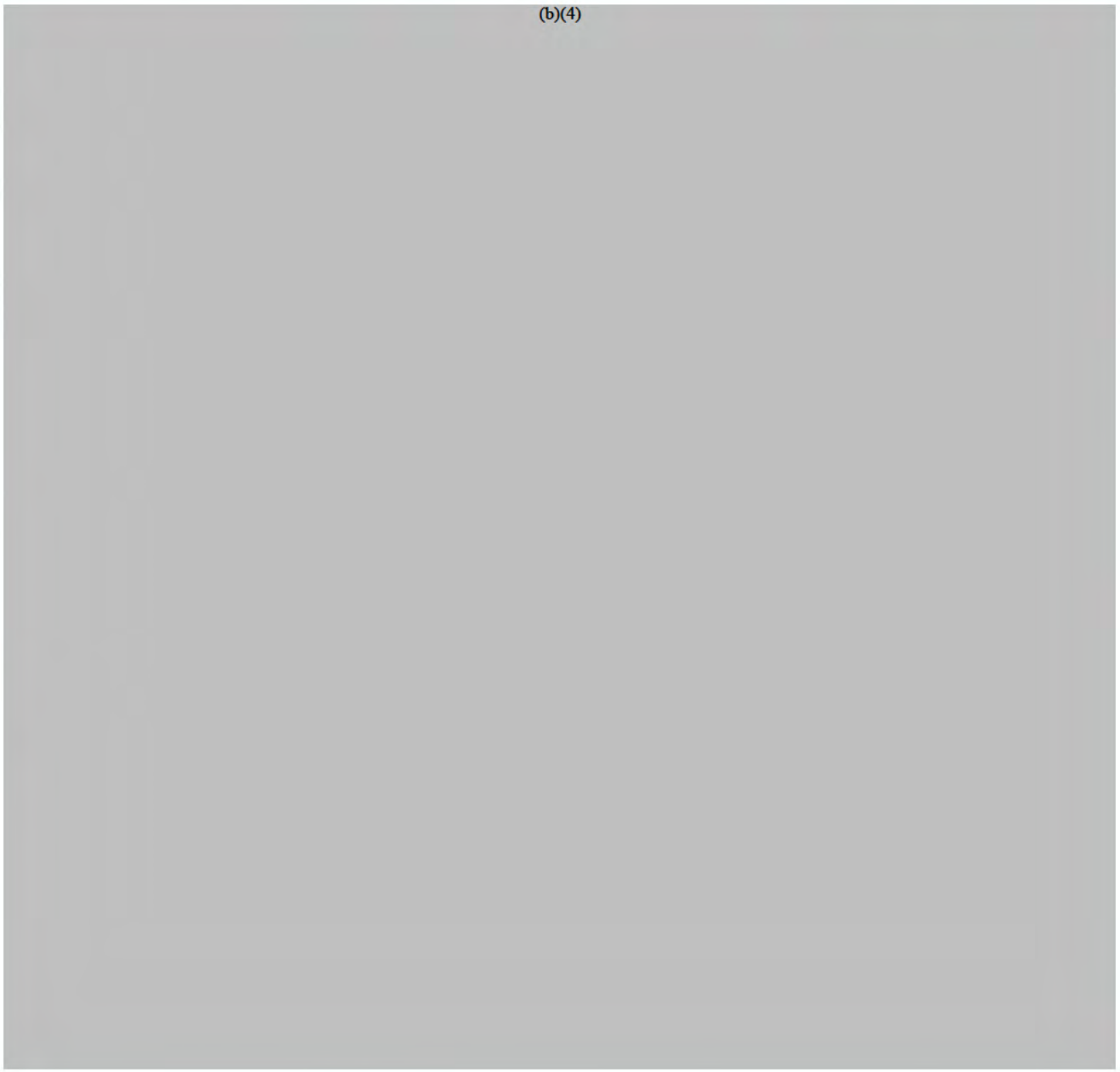

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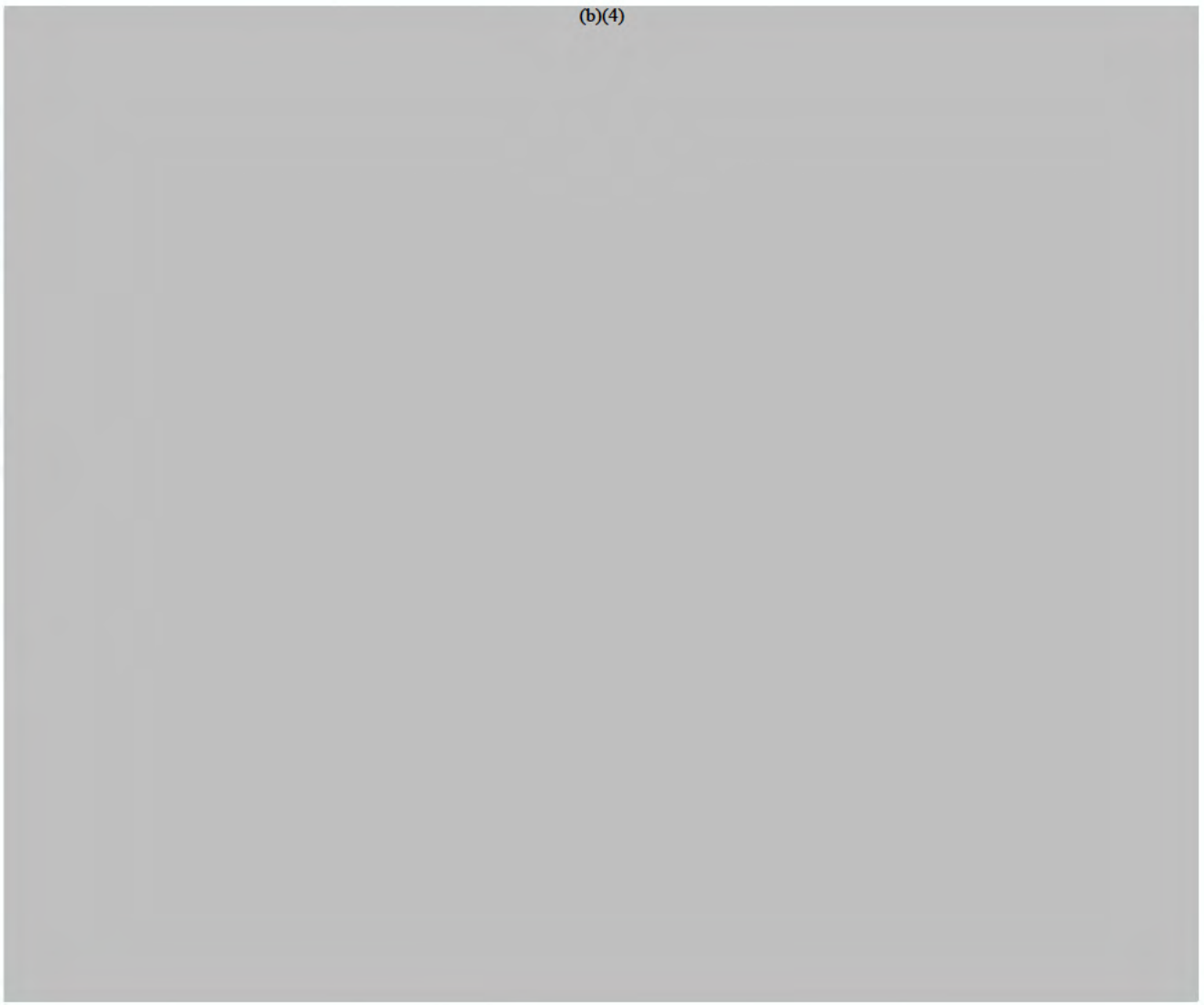

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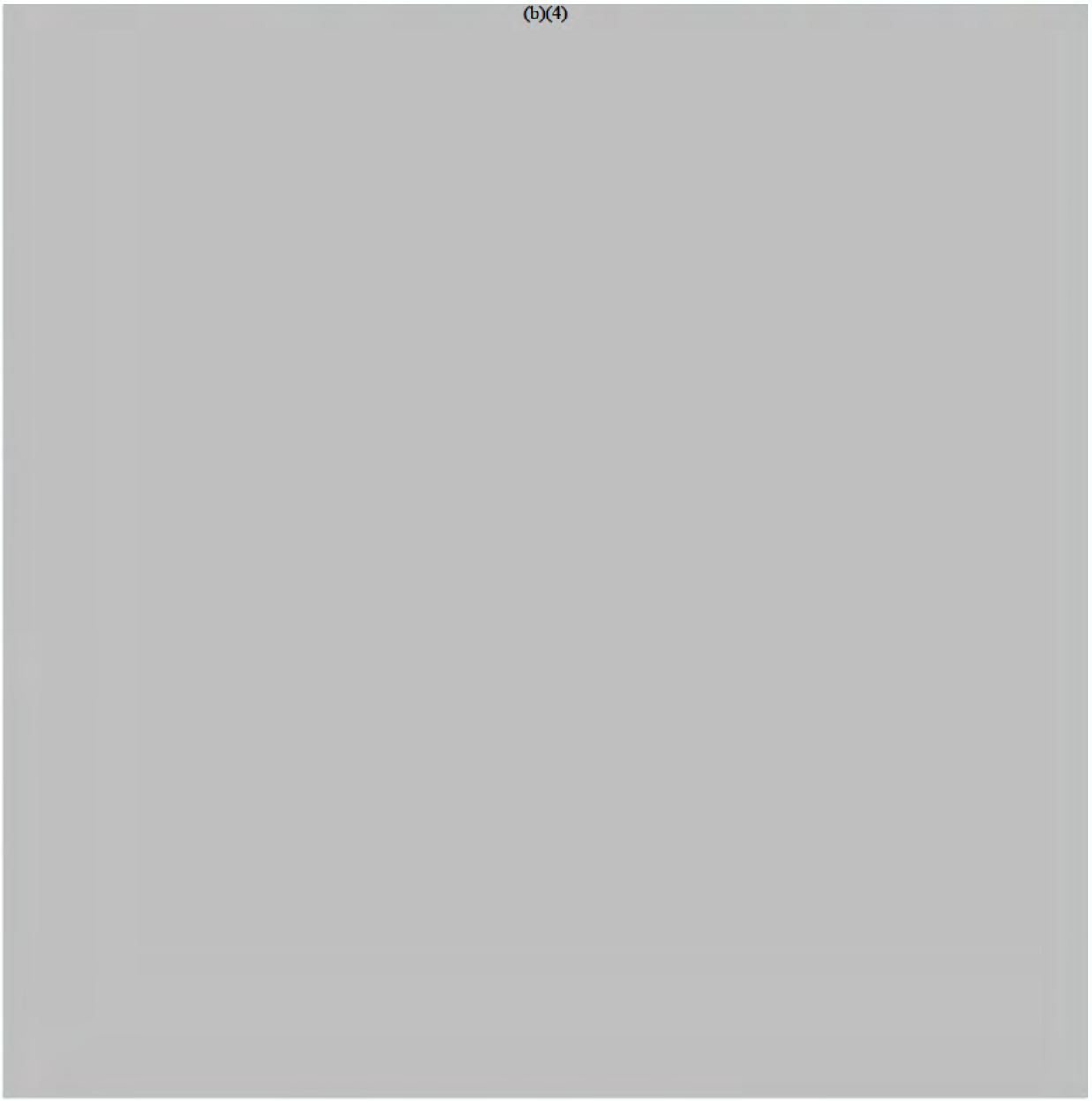

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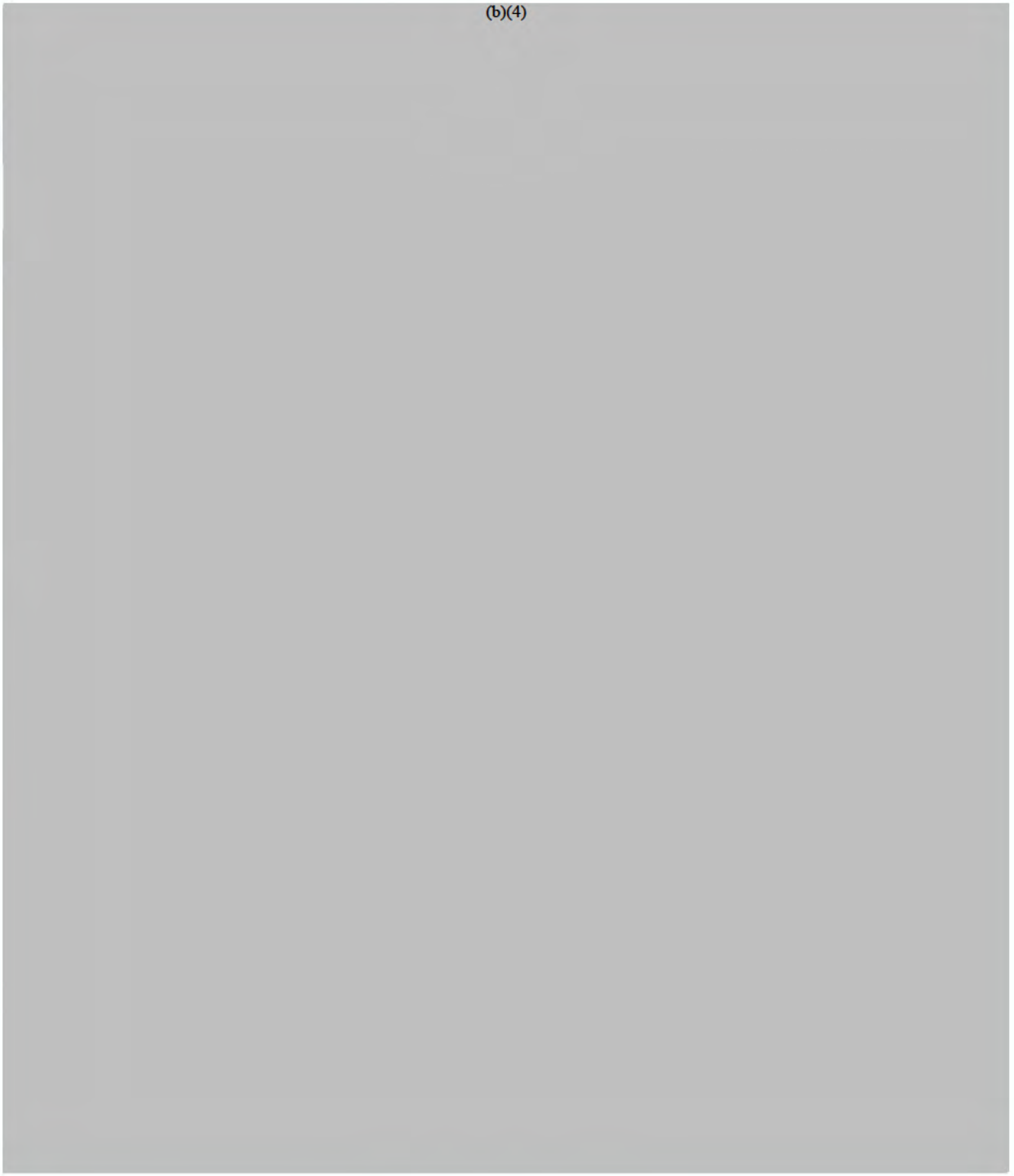

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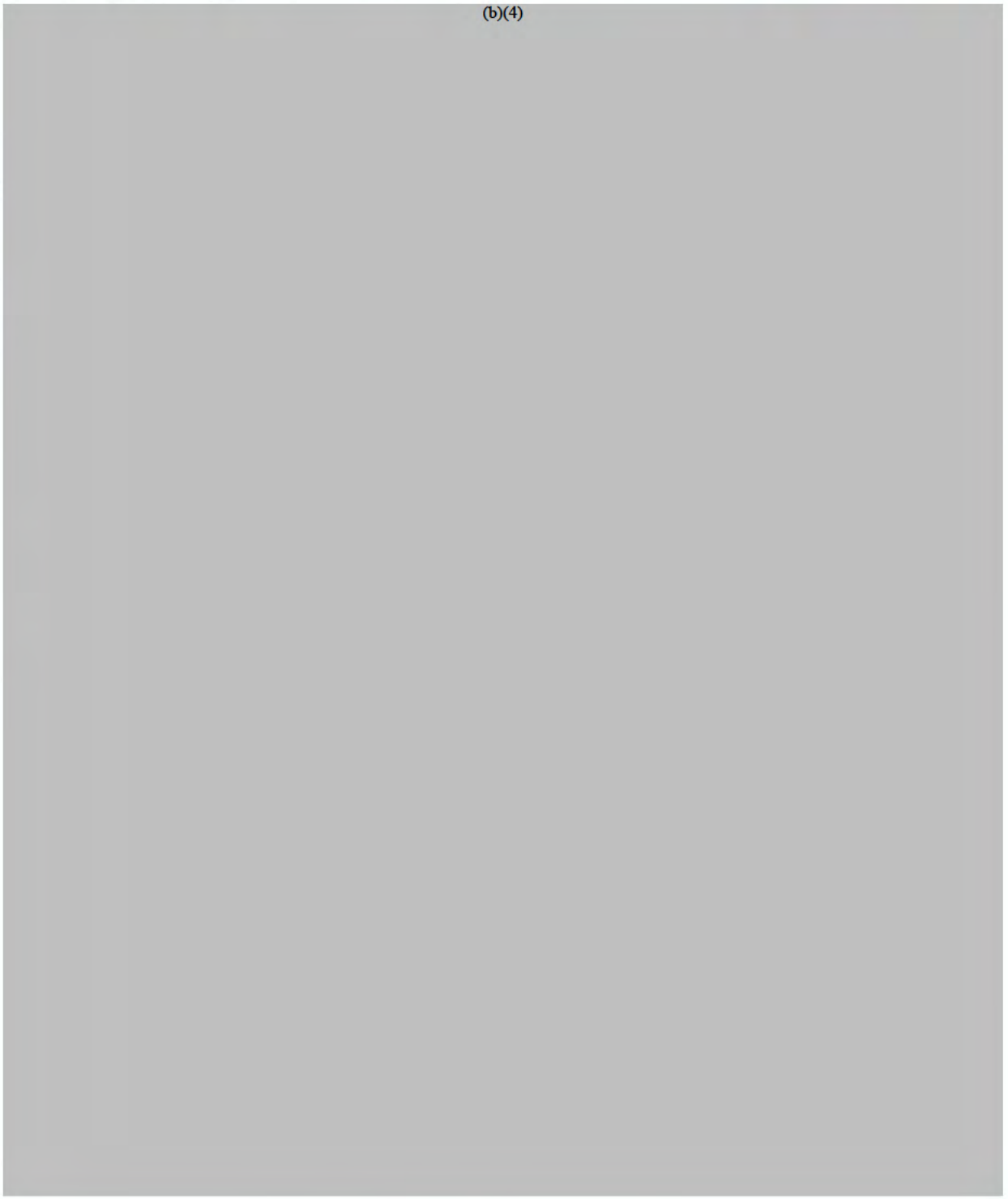

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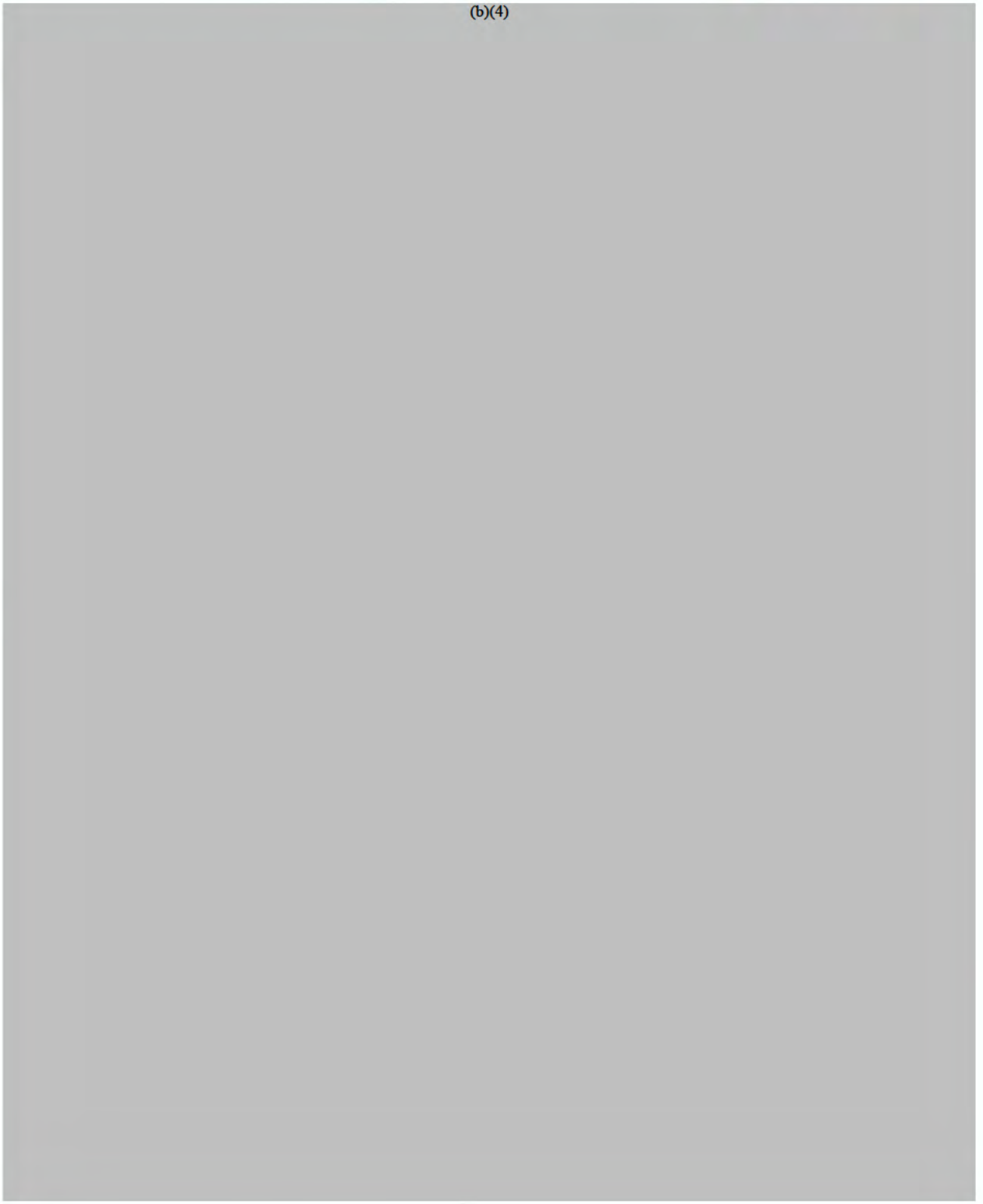

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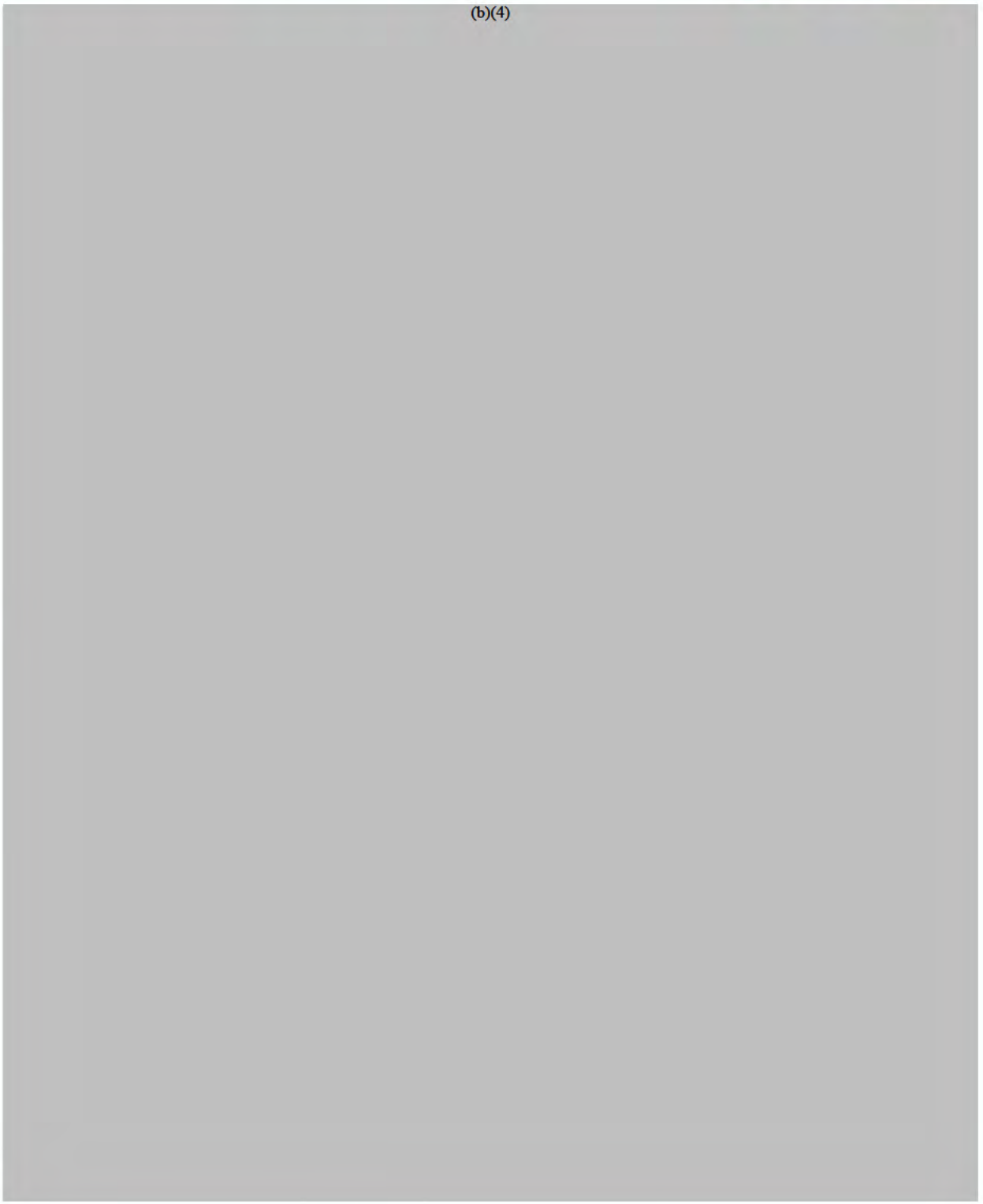

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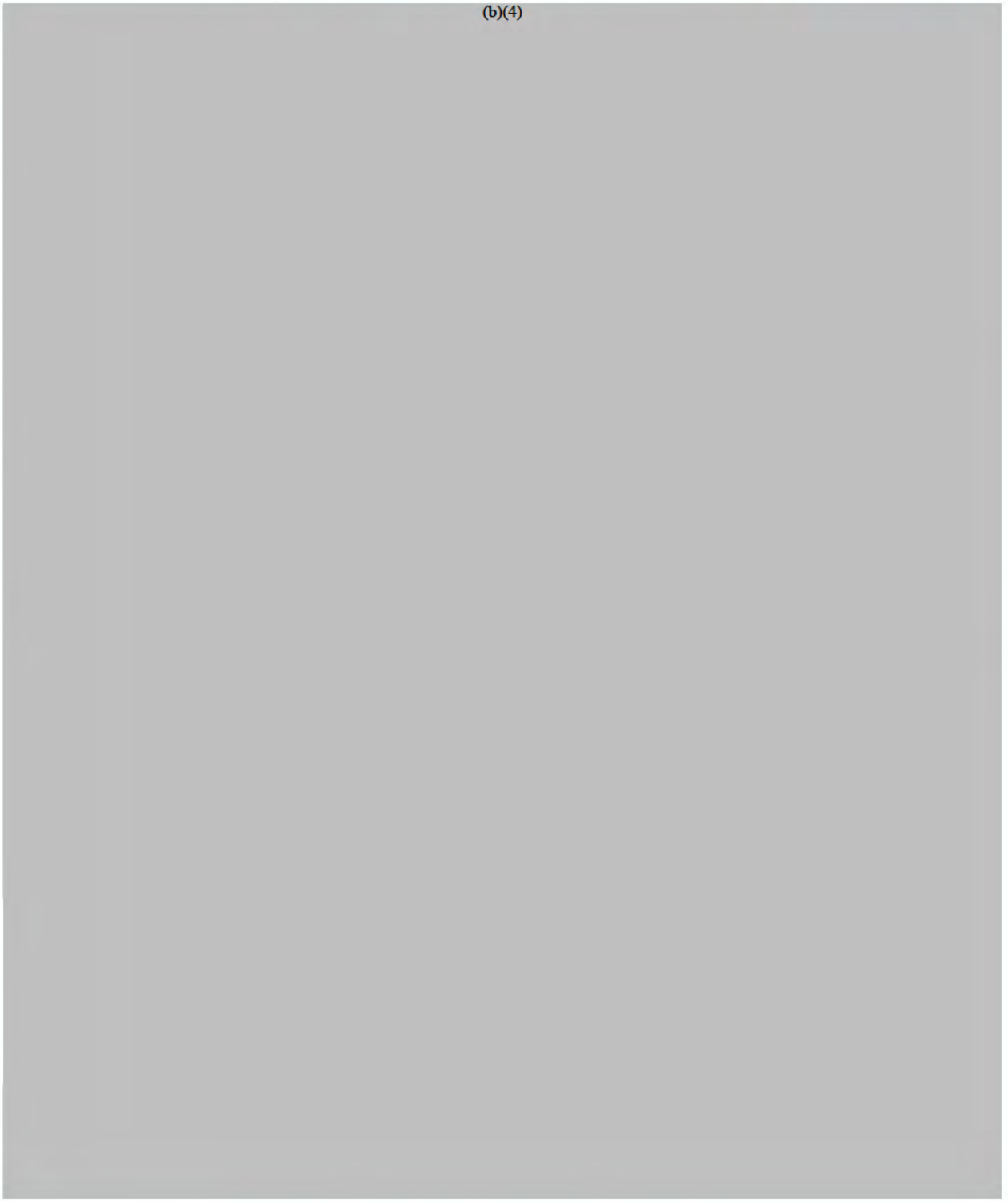

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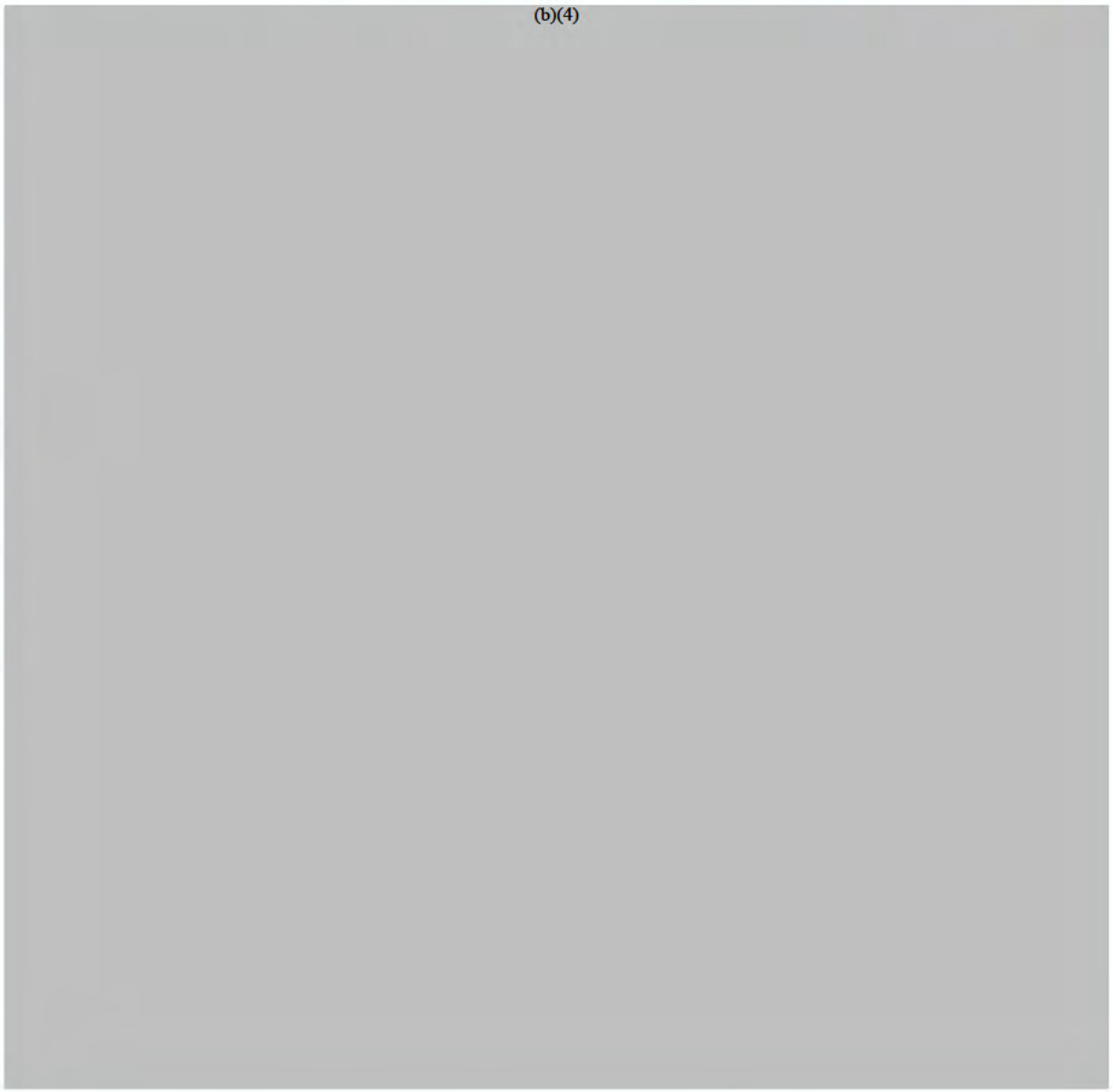

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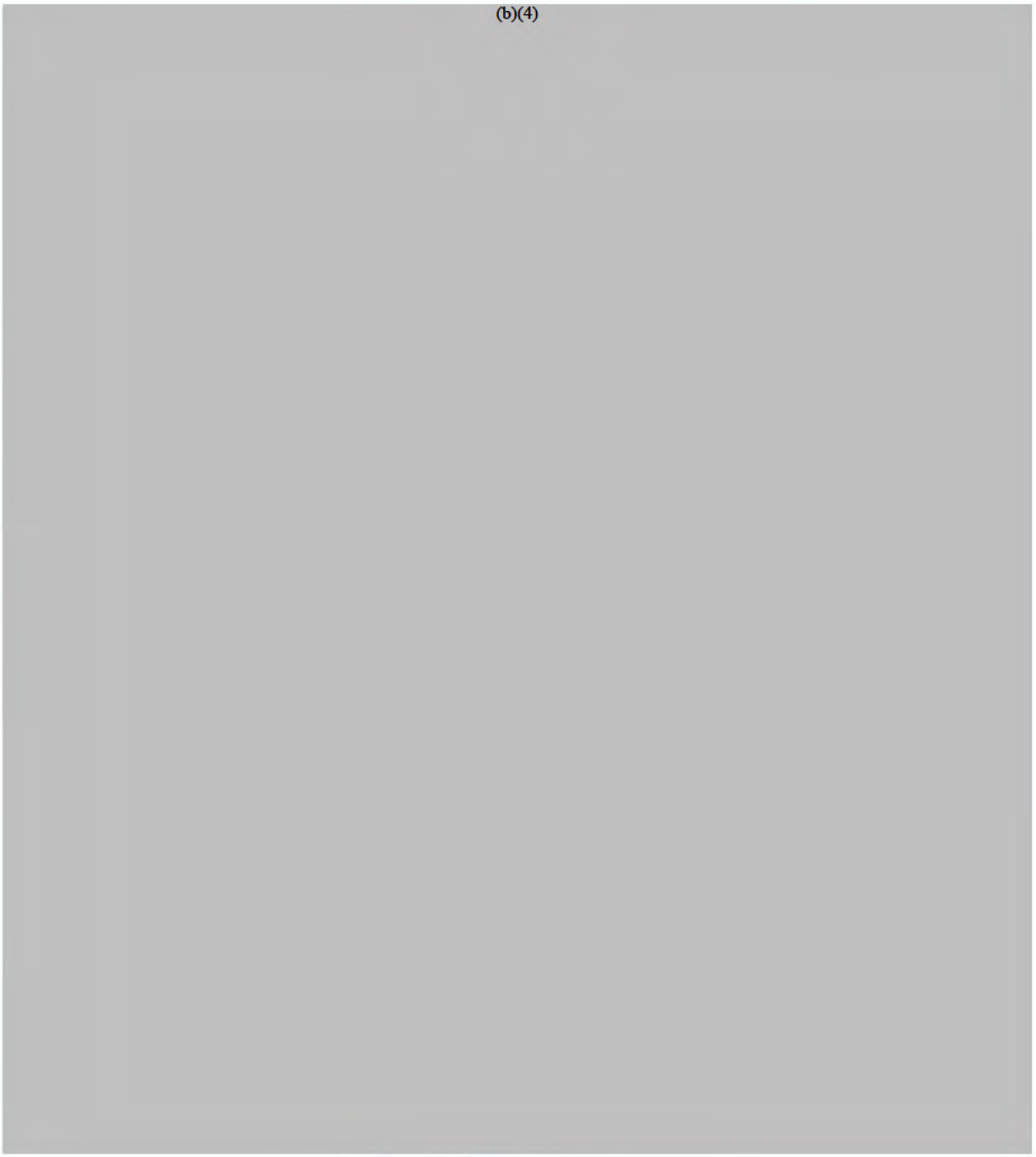
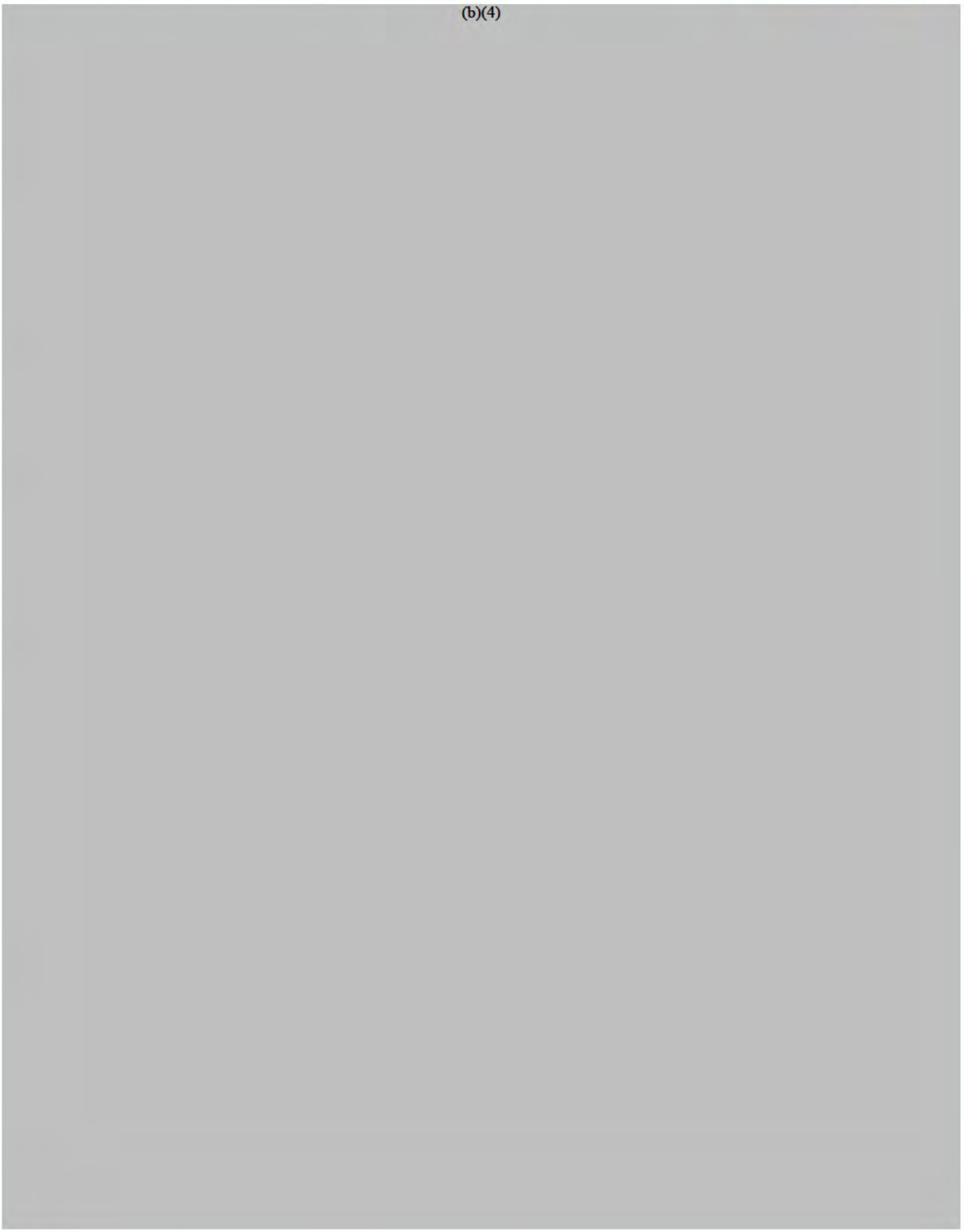


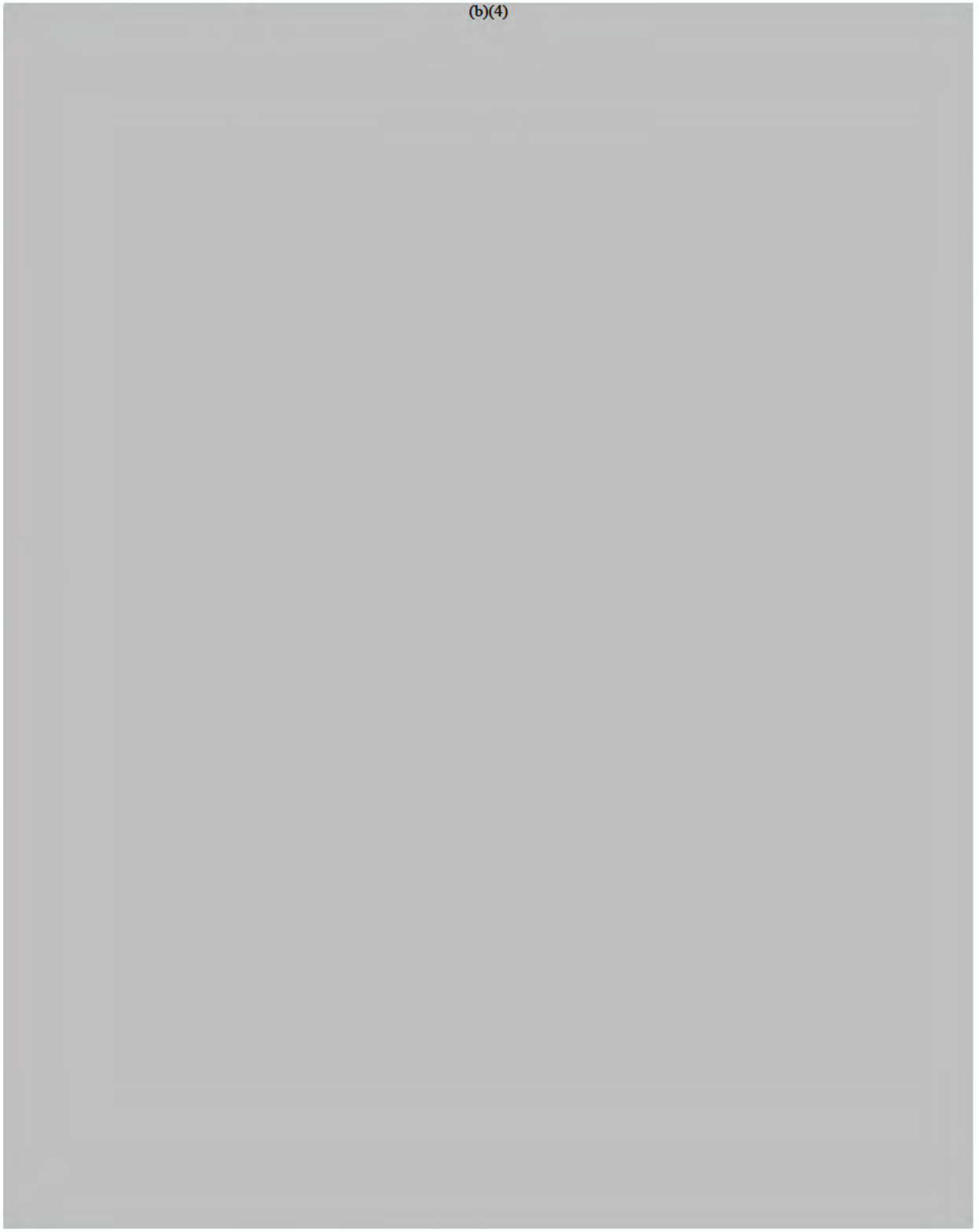
(b)(4)



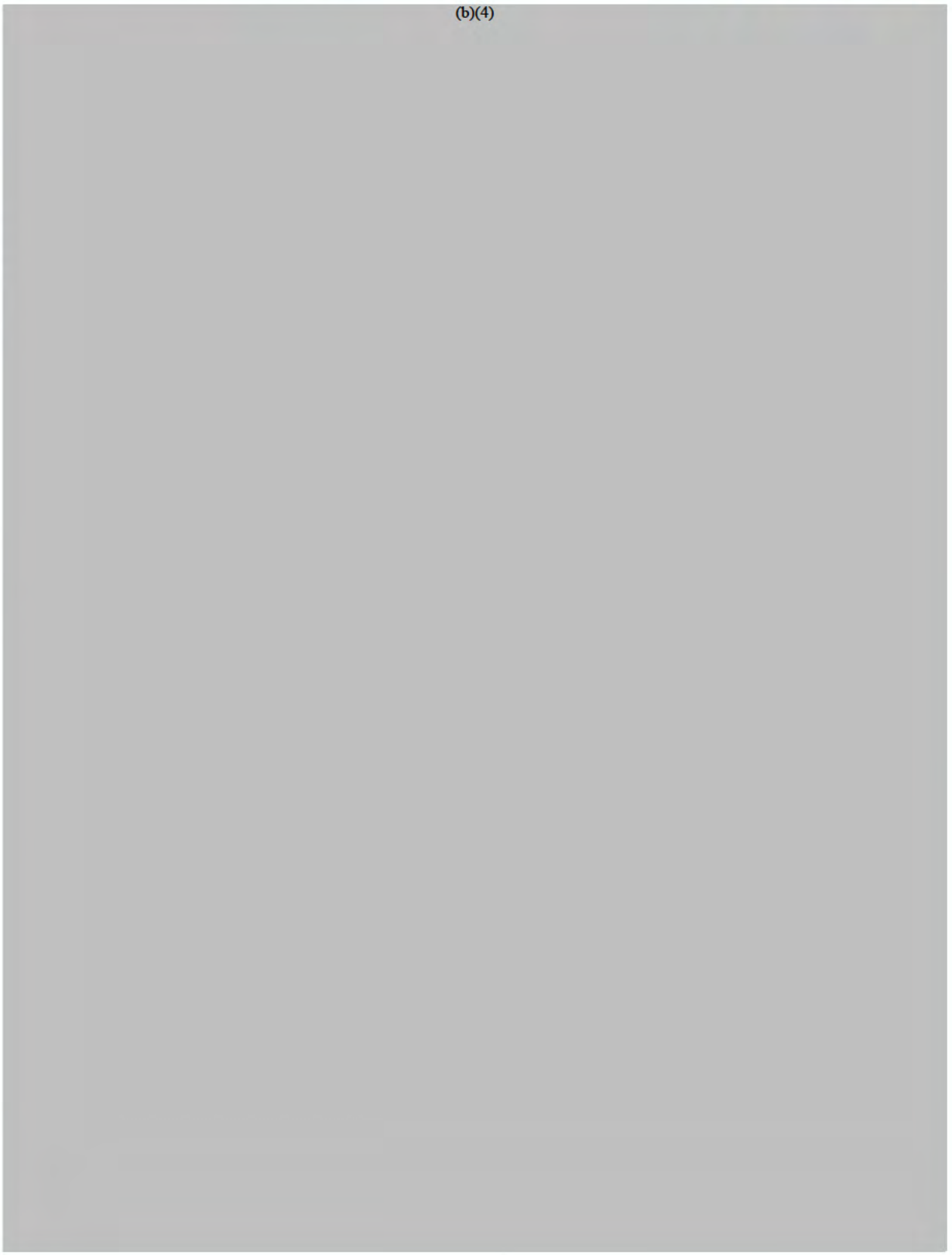
(b)(4)

3. Manufacturing

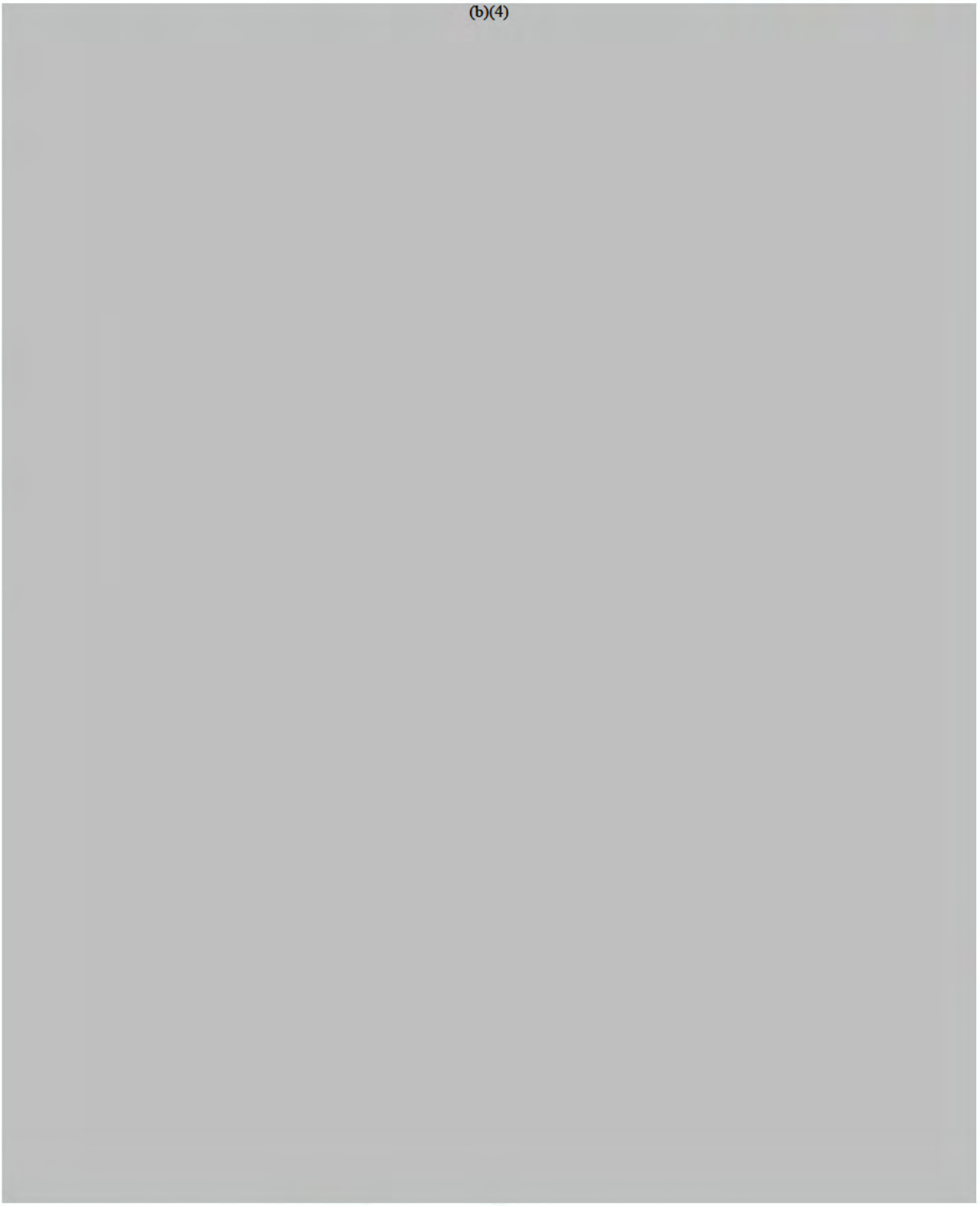
(b)(4)



(b)(4)



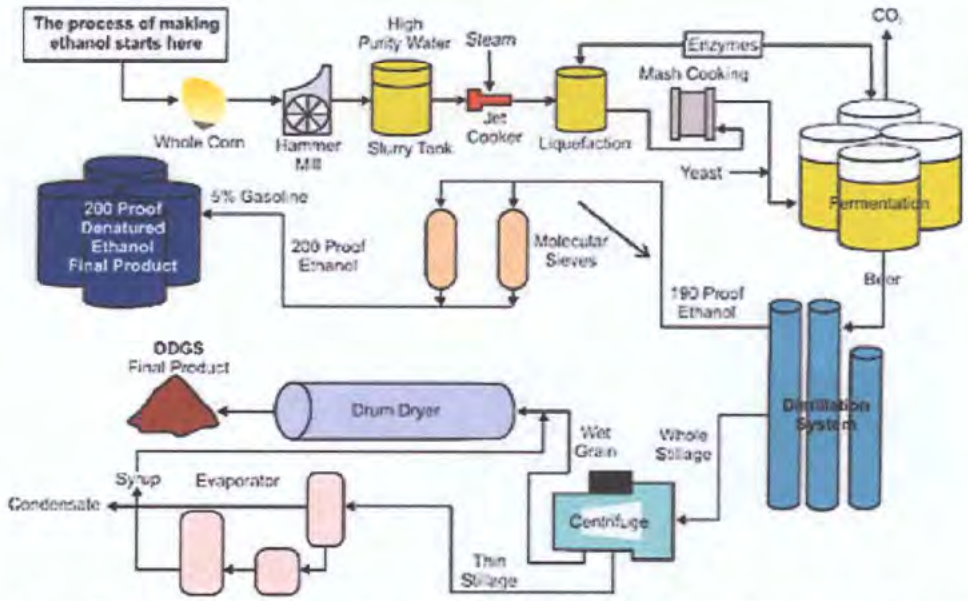
(b)(4)



3.2 Production of the DDG/S containing the Modified *Saccharomyces cerevisiae*

The Modified *Saccharomyces cerevisiae* is designed for the optimal production of bio-ethanol. The inactivated yeast is recovered from the stillage of the bio-fuel plant along with the unfermented plant matter in the same manner as traditional fermentation processes as illustrated in the process flow below.

Figure 2-13 Manufacture of the commercial yeast sold to ethanol plants



(Committee on Water Implications of Biofuels Production in the United States 2008)

3.3 Inactivation of the yeast

Under the current processing methods followed in a fuel ethanol facility, the Modified *Saccharomyces cerevisiae* will be exposed to lethal conditions in the distillation process step ensuring that only non-viable cells are incorporated into the feed of animals. Ethanol boils at 78.4^0 C at normal atmospheric pressure. **Annex 6** contains the results of experiments that confirm that the organism is destroyed at 80^0 C within 5 minutes. The yeast will be in the distillation pot for a much longer time as the entire fermentation batch is distilled prior to transferring the stillage to the recover steps. **Annex 7** contains additional experimental data showing the complete inactivation of the yeast at temperatures below temperatures used in the distillation step. The references Álcohol Textook, 'chapters 16 and 19' (Richards 2009, Earnest, Snyder et al. 2009) provided additional information regarding fermentation plant operations that support the premise that the yeast will be killed during downstream processing at the biofuel plant. On pg 279 in chapter 19 it is noted that '*The maximum temperature recommended for most strains of Saccharomyces cerevisiae is 35°C (at least in a stress-free environment)*

(Kelsall and Lyons, 2003). As temperatures climb above 35°C, vital proteins within the cell become structurally altered (denatured), causing a decrease in metabolic activities.'

3.4 The commercial product

3.5 Composition

The Modified *Saccharomyces cerevisiae* is composed of proteins, lipids, carbohydrates, cellulose and minerals. The commercial product for bioethanol production will be sold as either a dry powder produced from a concentrated liquid or the concentrated liquid itself (commercially referred to as 'cream yeast') by DSM Bio-based Products & Services. The inactivated yeast will be a component of Dried Distillers' Grains with or without Solubles (DDG/S) or sold alone as dry yeast.

3.5.1 Specifications

Certificates of analysis for typical Modified *Saccharomyces cerevisiae* batches demonstrate consistency of production lots to specifications (see **Annex 8**).

Table 3-1 Modified *Saccharomyces cerevisiae* liquid concentrate specifications and batch to batch variation

Parameter	Units	Specification (cream yeast)	Pilot Batch Rpt 1202 CRY	Batch C177000	Batch C177001
Appearance		Turbid cream yeast (concentrated broth) with a white/beige color	complies	complies	complies
Dry matter	%	15-20	(b)(4)		
pH		3.8-4.5			
Glucose	g/l	≤ 0.5			
Microbial contamination: total bacterial count	CFU/mL	≤ 10^6			
Microscopic observation in the EOF broth		conform	complies	complies	complies

3.6 Relevant properties

Physical properties of the dry form of Modified *Saccharomyces cerevisiae* are presented in Table 2-18.

Table 2-18 Relevant Physical and Chemical properties of Modified *Saccharomyces cerevisiae*

Properties	Value	Method / Data / Reference
Physical state	Free-flowing granules	Visual
Color	Tan	Visual
Yeast Dry Matter	(b)(4)	Vacuum oven @ 65°C
Moisture		By calculation
(b)(4)		Formulation calculation
	(b)(4)	

3.7 Stability

The Modified *Saccharomyces cerevisiae* has been developed for use in the fermentation of biomass-derived carbohydrates for the purpose of producing fuel ethanol. The organism is not designed for nor is it expected to be stored for long periods of time before use in the biofuel plant. The following information is provided only as an indication of stability as it affects the biofuel processor and not the stability of the inactivated cells present in the Distiller Dried Grains with or without solubles (DDG/S), or dried yeast products that could contain the Inactivated Modified *Saccharomyces cerevisiae* added to the feed of animals.

A pilot plant batch of yeast was split into smaller portions packaged in vacuum sealed, foil lined laminated pouches and stored at two temperatures. Pouches were removed from the storage chambers and an aliquot of yeast was either rehydrated first or added directly to the fermentation broth. Samples were taken from the fermenters at the prescribed times via an auto sampler, the optical density was measured and recorded and this value was used to calculate the cell concentration.

Table 2-19: Biomass in g/L at three fermentation times after storage at 4 and 30 °C

	Storage 4º C				Storage 4º C		
	12 hours	24 hours	36 hours		12 hours	24 hours	36 hours
	rehydrated before use				direct pitch		
Week 0	1,8	3,9	4,1				
Week 7	1,9	3,6	3,7				
Week 9	1,9	3,9	3,1				
Week 17	1,8	3,7	3,9		1,0	3,8	4,0
Week 26	1,0	4,3	4,2		0,2	2,2	4,3
Week 52	nd	nd	nd		0,1	0,1	0,2
	Storage 30º C				Storage 30º C		
	12 hours	24 hours	36 hours		12 hours	24 hours	36 hours
	rehydrated before use				direct pitch		
Week 0	1,8	3,9	4,1				
Week 7	1,7	3,6	3,6				
Week 9							
Week 17					0,3	2,4	4,1
Week 26	0,4	2,8	4,1		1,3	3,9	4,2
Week 52					0,1	0,1	0,1

The data shows that active Modified *Saccharomyces cerevisiae* retains its fermentative capacity after 6 months at 4° C, the recommended storage temperature. Similar results have been found for wine yeast (Simpson & Tracey 1986).

3.8 Nutritional Properties

Two pilot plant batches and a sub-sample of one commercial batch of Inactivated Modified *Saccharomyces cerevisiae* were sent to a third party commercial lab for nutritional analysis. A sample of a commercial baker's yeast was included for comparison because baker's yeast has long been an accepted ingredient in animal feed due to its nutritional properties. In addition, these nutritional analyses were compared to the nutritional data of active dry baker's yeast as published by the USDA.

Complete reports of the performed nutritional analyses are in **Annex 9**

Table 2-20 Nutritional properties of the active Modified *Saccharomyces cerevisiae*

Parameter	Units	Leavening agent, active dry baker's yeast*	Lot 1	Lot 2	Lot3	Baker's yeast (b)(4)
			1315 A	K160 A	1313 EF	
Dry Matter	%	94.9	92.2	92.1	89.7	95.9
Energy	kcal/Kg	3250	3218	3253	3145	3405
Protein	%	40.44	46.10	46.15	46.01	44.09
Fat	%	7.61	6.16	6.09	5.77	4.17
Crude Fiber	%	26.9**	<0.2	<0.2	<0.2	<0.2
Ash	%	NA	9.06	7.85	8.10	4.57
Ca	%	0.03	0.005	<0.004	0.004	0.09
P	%	0.637	1.88	1.54	1.33	0.79
K	%	0.955	2.26	2.54	2.16	1.55
Mg	%	0.054	0.27	0.18	0.19	0.09
Zn	ppm	79	121	106	93	80
Mn	ppm	NA	8	7	11	5
Cu	ppm	NA	7	9	12	2
Fe	ppm	22	49	11	68	54

NA: not available

* USDA National Nutrient Database for Standard Reference Release 26

** Total dietary fiber

The Modified *Saccharomyces cerevisiae* is derived from the same genus and species as baker's yeast. The nutritional composition of Modified *Saccharomyces cerevisiae* is therefore expected to be very comparable to that of dry baker's yeast already on the market. This was confirmed by comparing the nutritional composition of three lots of Modified *Saccharomyces cerevisiae* with the composition of active dry baker's yeast obtained from the USDA National Nutrient Database (USDA 2013), and the commercialized baker's yeast (b)(4) (see Table 2-20 above).

The difference in fiber content is due to methods of expressing fiber. In the active dry baker's yeast reported on the USDA database, the total dietary fiber content was determined, whereas only crude fiber content was measured in Modified *Saccharomyces cerevisiae* and (b)(4) baker's yeast.

Ash content is driven by the media composition. Baker's yeast has been commercially produced for over 75 years and the media composition has been optimized to be the least expensive formulation that allows for maximum cell mass in the shortest period of time. The media formulation for the Modified *Saccharomyces cerevisiae* has not been optimized at this point in

time and may be too rich in minerals. DSM anticipates that the concentration of the media components may be altered in the future.

The difference in protein is driven by differences in the parent strain, media composition and the differences in the fermentation process. The Modified *Saccharomyces cerevisiae* has been developed to efficiently perform in biofuel substrates such as crude corn digests. Baker's and brewer's yeast used in feed are valued for their protein content and an increase in protein would be welcomed by the feed industry.

High protein levels (up to 60% on dm is possible) lead to high yeast activity in bread applications, however they lead to a lower shelf life of the (fresh) yeast. In the case of dry yeast, an additional factor is that high protein yeast (b)(4).

All in all, the yeast protein in baker's yeast products may vary from 40% to 60-65% on dm. For active dry yeast it may vary from 40-55% on dry matter (Reed, Peppler 1973).

The specification in the 2013 AAFCO OP definition 96.1 for dried yeast (Association of American Feed Control Officials 2013) is that the organism is *Saccharomyces cerevisiae* and has a minimum protein content of 40%. The Modified *Saccharomyces cerevisiae* easily meets this requirement.

The slightly low dry solids content for sample 1313EF presented by the outside laboratory is inconsistent with the sample analysis at the time of manufacture, see Annex 10, were the plant QC department recorded a dry solids of 92.2% which was within specification when the sample was taken for shipment to the analytical laboratory. Yeast is a hydroscopic substance and short exposure to high humidity, such as a rainy day, can quickly affect the total moisture of an open container. We suspect that during shipment or handling at the analytic lab the sample gained moisture.

Yeast is utilized as an animal food ingredient for its nutritional composition, the Modified *Saccharomyces cerevisiae* has a nutritional composition that is substantially similar to traditional yeast utilized in feed. Feed manufacturers use sophisticated nutritional formulation programs to produce batches of feed and adjust each batch based upon the compositional make-up of the ingredients. This allows the feed manufactures to compensate for ingredient availability and seasonal variation in composition. Any difference between the Modified *Saccharomyces cerevisiae* and for example a Baker's yeast in protein level would be easily managed by a feed mill nutritionist.

4. Dried Distillers Grains

The inactivated Modified *Saccharomyces cerevisiae* may become a component of distillers dried grains (DDG) in the same manner as traditional yeast used in grain fermentations and the genetically engineered *Saccharomyces cerevisiae* produced by the Mascoma Corporation that was listed by AAFCO after CVM review. DSM asked a potential customer who produces bio-fuel to evaluate the DSM Modified *Saccharomyces cerevisiae* in their process and to provide detailed analyses of the resultant distillers products. Three batches of Distillers Dried Grains with Solubles (DDGS) containing the Inactivated Modified *Saccharomyces cerevisiae* were produced via fermentation of #2 yellow corn suitable for use for animal feed in 2012 following the commercial bio-fuel producer's current process. Corn is the most common substrate for bio-ethanol production with about 5 billion bushes being utilized for fuel in 2013. (ERS 2014)

Table 4-1 Ingredients used in the bio-fuel fermentation

Input	US Approval
	(b)(4)

Input	US Approval
(b)(4)	

The product data sheets for each ingredient are in Annex 11.

4.1 Comparison of DDGS produced with conventional and Modified *Saccharomyces cerevisiae*

Four samples of DDGS produced with a conventional ethanol production yeast and four with the DSM Modified *Saccharomyces cerevisiae* were produced in a potential customer's plant using #2 yellow corn suitable for use for animal feed as the fermentation substrate. The samples were analyzed in the plant's laboratory using AOCS or AOAC methods as applicable.

Table 4-2 Gross composition

	Method		AOCS Ba 6a-05	AOCS Am 5-04	AOAC 990.03	AOAC 979.10	AOAC 942.05	NFTA 2.2.2.5
Sample	Yeast	Oil Reduced	% Crude Fiber (Dry Basis)	% Fat (Dry Basis)	% Protein (Dry Basis)	%Starch (Dry Basis)	% Ash (Dry Basis)	% Total Solids
503	Modified *Saccharomyces cerevisiae*	No	5.49	9.90	45.11	0.49	4.10	93.68
507	Modified *Saccharomyces cerevisiae*	No	5.45	10.72	41.83	2.27	3.08	93.48

	Method		AOCS Ba 6a-05	AOCS Am 5-04	AOAC 990.03	AOAC 979.10	AOAC 942.05	NFTA 2.2.2.5
Sample	Yeast	Oil Reduced	% Crude Fiber (Dry Basis)	% Fat (Dry Basis)	% Protein (Dry Basis)	%Starch (Dry Basis)	% Ash (Dry Basis)	% Total Solids
598	Modified *Saccharomyces cerevisiae*	No	5.84	10.29	46.08	1.70	3.13	93.90
599	Modified *Saccharomyces cerevisiae*	No	5.74	11.70	42.37	2.34	2.85	92.95
908	Conventional *Saccharomyces cerevisiae*	Yes	Not Tested	9.46	29.91	7.19	5.85	90.81
923	Conventional *Saccharomyces cerevisiae*	Yes	7.92	7.65	29.68	9.86	2.74	92.83
928	Conventional *Saccharomyces cerevisiae*	Yes	Not Tested	9.98	28.56	9.45	7.71	87.47
924	Conventional *Saccharomyces cerevisiae*	Yes	5.32	9.04	29.37	11.6	4.77	89.88

Discussion

The primary difference in the nutrient composition of the DDGS resulting from the use of the Modified *Saccharomyces cerevisiae* is an increase in protein concentration. This is due to the fact the Modified *Saccharomyces cerevisiae* ferments xylose thus reducing the amount of carbohydrate solids in the DDGS. A fermentation using conventional yeast has a higher percentage of carbohydrates because the traditional yeast cannot ferment pentoses such as xylose and arabinose, which can be up to 2.75 percent of the carbohydrates in corn remains in the DDGS. See Berger 2006 and Hromadkova & Ebringerova 1995.

4.1.1 Amino Acids

The primary nutrient provided by yeast is protein. Two factors affect the nutritional value of a protein, the amino acid profile and the digestibility of the protein molecules. A more detailed analysis of the amino acid profile and digestibility were performed by the University of Illinois under the direction of Dr. Carl Parsons.

Annex 12 contains the data tables from the laboratory and the statistical analysis.

Table 4-3 DDG amino acid profile comparison, % of protein (dry basis)

Amino Acid	Average Modified Yeast	Average Conventional Yeast	Std Deviation Modified Yeast	Std Deviation Conventional Yeast	P value
ASP	1.88	1.97	0.106	0.105	0.249
THR	1.10	1.16	0.054	0.049	0.209
SER	1.40	1.33	0.084	0.063	0.277
GLU	4.90	4.20	0.352	0.521	0.067
PRO	2.36	2.12	0.134	0.225	0.122
GLY	1.10	1.27	0.070	0.064	0.011
ALA	2.17	2.10	0.112	0.126	0.457
CYS	0.55	0.54	0.026	0.042	0.714
VAL	1.50	1.61	0.084	0.124	0.196
MET	0.64	0.60	0.039	0.057	0.390
ILE	1.18	1.19	0.058	0.067	0.856
LEU	3.79	3.46	0.182	0.285	0.099
TYR	1.22	1.16	0.070	0.083	0.297
PHE	1.56	1.60	0.081	0.141	0.639
LYS	0.75	0.88	0.077	0.067	0.055
HIS	0.72	0.82	0.056	0.041	0.027
ARG	1.18	1.47	0.101	0.107	0.056
TRP	0.21	0.25	0.031	0.018	0.205

Conclusion

There is no statistically significant difference in the amino acid profile of the DDGS produced using the Modified *Saccharomyces cerevisiae* and the conventional yeast with the exception of glycine and histidine, two non-essential amino acids. Therefore the Modified *Saccharomyces cerevisiae* is substantially similar to the conventional yeast already being used as a feed ingredient.

4.1.2 Digestibility

The same samples that were analyzed for the amino acid profiles were also evaluated for digestibility in roosters.

Rooster feeding trials were performed by Dr. Carl Parsons – University of Illinois. Mature roosters that were cecetomized were used to determine digestibility. Statistics are in Annex 13.

- Adult leghorn cecectomized males were used.
- Roosters were fasted for approximately 27 hours.
- Each sample was fed to 4 roosters and the excreta from each rooster was pooled by weight after drying.
- Roosters were fed approximately 25g.
- Roosters were then placed in cages with ad lib water access with a collection tray underneath for 48 hours.
- Excreta were collected from the trays 48 hours after feeding. Excreta were collected into pre-weighed freezer containers using water.
- Excreta were freeze dried and weighed. Then the excreta were ground using a standard small coffee grinder.
- As pooled samples were used, the pool was weighted so that the rooster with the most contributed the most to the pool.
- The feed and pooled excreta were sent to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories for Amino Acid analysis. AOAC Official Method 982.30 E(a,b,c), chp. 45.3.05, 2006. http://www.aescl.missouri.edu/MethRefs.html
- After results were received, the values were placed into a spreadsheet using an endogenous correction (AA values for fasted rooster excreta) and then the percent of each amino acid that was digested was determined.

Table 4-4 DDGS amino acid digestibility

Amino Acid	Average Modified Yeast	Average Conventional Yeast	Standard Deviation Modified Yeast	Standard Deviation Conventional Yeast	P value
ASP	72.87	69.17	2.23	3.71	0.249
THR	73.26	71.16	2.75	2.46	0.209
SER	79.22	75.53	2.52	5.29	0.277
GLU	84.16	80.75	0.89	3.93	0.066
PRO	80.79	79.68	1.26	3.62	0.122
ALA	84.16	81.39	1.15	2.78	0.0578
CYS	68.62	67.73	1.67	4.93	0.3727
VAL	78.43	77.23	2.26	2.21	0.2370
MET	85.64	82.20	1.18	2.92	0.0357
ILE	79.58	77.95	2.42	1.85	0.1626
LEU	86.99	86.72	1.08	1.54	0.3927
TYR	86.69	82.47	1.75	2.45	0.0156
PHE	84.60	82.74	1.56	1.64	0.0758
LYS	57.11	50.44	5.41	6.59	0.0844
HIS	75.83	75.54	1.68	3.26	0.4398
ARG	84.29	82.30	2.68	2.38	0.1551
TRP	87.97	87.94	1.79	2.90	0.4927

Conclusion

There is no statistically significant difference in digestibility between the Modified *Saccharomyces cerevisiae* and the conventional yeast except for methionine and tyrosine which may be due to three samples of the conventional yeast DDGS having both methionine and tyrosine digestibility well below the fourth sample which in turn was similar to the level of the modified yeast samples. Laboratory technician error may be the cause for the difference.

4.1.3 Metabolizable Energy

Total Metabolizable Energy (TME) was determined by Dr. Carl Parsons – University of Illinois using intact roosters.

- The feed and pooled excreta were sent to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories for nitrogen (N) analysis. Combustion Analysis (LECO) AOAC Official Method 990.03, 2006. http://www.aescl.missouri.edu/MethRefs.html
- Dry matter (DM) of feed was performed in the Animal Science Laboratory - University of IL. Approximately 2g of feed is placed in a pre-weighed beaker and the total weight is recorded. Feed is dried at 105C for 24 hours, allowed to cool for about 30 minutes, then weighed.
- Gross energy (GE) was performed in the Animal Science Laboratory - University of IL with a Parr 1261 bomb calorimeter. GE was performed on the feed, as-is, and the pooled excreta.
- The GE of feed and excreta, the N of feed and excreta, and the DM of feed were placed into a spreadsheet where endogenous values again were used to determine the TME of the feedstuff.

Table 4-5 Metabolizable Energy

Sample	ID	Oil Reduced	Gross Energy of feed as-is(kcal/g)	Dry Matter Percent	TME_n (kcal/g DM)
503	Modified *Saccharomyces cerevisiae*	No	5.10	91.70	2.89
507	Modified *Saccharomyces cerevisiae*	No	5.14	92.50	3.09
598	Modified *Saccharomyces cerevisiae*	No	5.17	93.10	2.92
599	Modified *Saccharomyces cerevisiae*	No	5.20	94.60	2.96
908	Conventional *Saccharomyces cerevisiae*	Yes	4.75	89.00	2.96

923	Conventional *Saccharomyces cerevisiae*	Yes	4.84	92.80	2.74
928	Conventional *Saccharomyces cerevisiae*	Yes	4.75	89.80	2.65
924	Conventional *Saccharomyces cerevisiae*	Yes	4.65	88.50	3.16

The average TME for Modified *Saccharomyces cerevisiae* DDGS is 2.965 kcal/g DM whereas the average TME for conventional yeast DDGS was 2.878 kcal/g DM. The observed higher for the Modified *Saccharomyces cerevisiae* value was most probably due to the higher level of lipids in the Modified *Saccharomyces cerevisiae* due to a processing constraint in the plant. None the less, the DDGS from the process using the Modified *Saccharomyces cerevisiae* is a suitable source of energy. If the oil is reduced in the commercial scale production, the nutritional value of the product will be communicated to customers who will use one of the feed formulation software packages to make the necessary adjustments to compensate for nutrient variability.

Conclusion

DDGS is a primary source of protein in modern feed formulation. Based upon the minimal difference in amino acid composition and amino acid digestibility, there is no significant difference between the proteins contained in the DDGS from a conventional yeast fermentation and that of Modified *Saccharomyces cerevisiae*. The increased protein concentration in the DDGS from the fermentation of grains with Modified *Saccharomyces cerevisiae* is due to a reduction in carbohydrates, which is the purpose of using the Modified *Saccharomyces cerevisiae;* converting more carbohydrates to ethanol. The total metabolizable energy of the DDGS from an ethanol fermentation using Modified *Saccharomyces cerevisiae* was greater than from conventional yeast fermentation primarily due to an up-steam processing difference that results in less lipids in the DDG from conventional yeast fermentation.

When all the nutritional properties of the DDGS produced from an ethanol fermentation process utilizing Modified *Saccharomyces cerevisiae* are evaluated, particularly when the availability of sophisticated feed formulation software is considered, it is apparent that the DDGS is suitable for use in animal food.

The above information is provided as an example of possible DDGS nutritional properties; the inherent variability in grain composition due to growing conditions at different locations, yearly weather patterns and fermentation practices may impact the nutrient content of DDGS from bioethanol fermentations which is out of the control of the submitter and would not be due to the use of the Modified *Saccharomyces cerevisiae*.

5. Information on any self-limiting levels of use

It is not expected that the amount of Inactivated Modified *Saccharomyces cerevisiae* in DDG/S or feed will be higher than the amount of classical *Saccharomyces cerevisiae* already found in feed or DDG/S, as a byproduct of conventional ethanol distillation. It was estimated that dried yeast represents 20% by weight of the dried distillers' solubles (Liu 2011).

The expected animal intake of DDG/S containing Inactivated Modified *Saccharomyces cerevisiae* is equal or less than the current intake of DDG/S containing classical *Saccharomyces cerevisiae* derived from starch-based biomass. These intakes depend on the nutritional needs of the specific target animals and are summarized in the table below (Table 3-1).
Since the nutritional content of Inactivated Modified *Saccharomyces cerevisiae* is very similar to conventional *Saccharomyces cerevisiae* (see Table 2-20), the intake levels of Inactivated Modified *Saccharomyces cerevisiae* by the target animals will not exceed what is expected from conventional *Saccharomyces cerevisiae*.

The following table illustrates the maximum percentage of modified yeast in the diet based upon the literature for DDG/S in the diet and the quantity of yeast in DDG/S (Liu 2011).

Table 5-1 Intake levels of DDG/S in feed of target animals

Species / Class	DDG/S Maximum % of Diet	Modified *Saccharomyces cerevisiae* Maximum % of Diet
Swine (Shurson, Spiehs et al.)		
Nursery pigs (>15 lbs.)	25	5
Grow-finish pigs	20	4
Developing gilts	20	4
Gestating sows	50	10
Lactating sows	20	4
Boars	50	10
Poultry (National Corn Growers Association 2008)		
Chicken - Broilers	10	2
Chicken - Layers	15	3
Turkeys (grow/finish)	15	3
Bovines (Weiss, Eastridge et al.)		
Beef cattle	25	5
Dairy cows	25	5

5.1 Spill-over Analysis

The primary difference between the engineered strain that is the subject of this GRAS dossier and the wild type *S. cerevisiae* is the ability of the engineered strain to efficiently convert xylose into ethanol. The mechanistic reason for the poor ability of *S. cerevisiae* to utilize xylose was report by Batt et al. in 1986. The xylose metabolic pathway is found in many strains of wild type yeast and the pathway is well understood. (Toivari et al. 2004). The parent strains of the GRAS substance were previously studied on three occasions to ascertain the possible production of unusual metabolic by-products when grown on a synthetic medium designed to mimic a plant carbohydrate hydrolysate. In 2004 two engineered strains were compared to the wild-type parent and the only significant difference in metabolite formation was that of an environmentally induced strain RWB202-AFX which produced xylitol whereas the engineered strain and the parent produced almost none, (Kuyper et al. 2004). Analysis of a strain engineered for improved xylose utilization was found to produce 0.5, 4 and 1.5 times as much glycerol, acetate and lactate respectively than the wild type; when the engineered strain was grown on a glucose, xylose mixture vs. the wild type which was grown on glucose. However, the increases in absolute concentration were quite small, < 10 mM and were not different from another strain produced by environmental induction, (Kuyper et al. 2005a). A similar experiment with another engineered strain showed reduced acid production (Kuyper 2005b). A review article by Kim et al. in 2013 noted that modifications of other strains in different laboratories had similar results when the goal is to increase ethanol production via pentose fermentation (Kim et al. 2013). A study of nine strains of *S. cerevisiae* engineered to ferment xylose via different alterations to the pathway revealed minimal production of xylitol on a synthetic media, yeast nitrogen base with 4% xylose.

At the request of CVM a new study was performed to determine if the results obtained with the parent strains in defined media would be the same with (b)(4) in a fermentation broth indicative of a corn hydrolysate found in bioethanol facilities. To that end, DSM obtained corn hydrolysate from a USA based bioethanol manufacturer and fermented the substrate simultaneously in six paired lab fermenters utilizing a wild type parent strain (b)(4), (b)(4) and a commercial yeast marketed for bioethanol fermentation, Ethanol Red. The corn hydrolysate was used at a concentration that the bioethanol manufacturer indicated was typical of that used in commercial production (see table 5.2 for sugar values in corn hydrolysate as determined by HPLC). The fermented broth was collected after 48 hours as is the common practice in commercial bioethanol manufacture. The broth was filtered to remove the yeast and other suspended solids and analyzed by HPLC with two methods to cover quantification for the same xylose metabolites as done in the previously published studies and for volatile acids as the latter were what CVM indicated were of concern for animal health if the DDG from a fermentation utilizing (b)(4) was fed to livestock (see Table 5.3 for metabolite levels at the sampling point at the end of fermentation [48h]). CVM was unable to provide any other direction regarding possible metabolites of concern for the agency. No attempt was made by DSM to identify other metabolites because as noted in section 2.3 of this dossier, and the literature, the optimization of the xylose metabolic pathway does not lead to the production of other metabolites.

The following tables provide the results of the analysis.

Table 5.2 Corn Hydrolysate Composition g/kg (oligosaccharides were not quantified)

	Total disaccharides	Glucose	Xylose	Arabinose	Glycerol	Furfural	Ethanol	HMF	Xylitol
Corn Hydrolysate (fermentation medium	6.9	23.3	22.4	12.0	1.5	0.5	0.5	0.1	<0.1
LoD (g/kg)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 5.3 Spill-over Analysis Results

Metabolite (g/kg)	Xylitol	Xylulose	Ribulose	Succinic acid	Lactic Acid	Formic Acid	Acetic Acid	Propionic acid	Butanoic acid
LoD (g/kg)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Corn Hydrolysate (fermentation medium) replicate 1	<	<	1,3	<	0,1	0,3	2,4	<	<
Corn Hydrolysate (fermentation medium) replicate 2	<	<	1.4	<	0,2	0,5	1,9	<	
TW2015-8A1.1-EtOH Red	<	<	1.4	<	0,1	0,3	2,4	<	<
TW2015-8A4.1 – EtOH Red	<	<	1.4	<	0,1	0,4	2,3	<	<
TW2015-8A3.1 – (b)(4)	<	<	1.4	<	0,2	0,4	2,2	<	<
TW2015-8A6.1 – (b)(4)	<	<	1.4	<	0,2	0,4	2,2	<	<
TW2015-8A2.1 – (b)(4)	<	<	1.4	<	0,1	0,4	2,2	<	<
TW2015-8A5.1 – (b)(4)	<	<	1.4	<	0,2	0,4	2,2	<	<

In summary, the study did not reveal any significant differences between the engineered yeast, the commercial yeast nor the wild type parent. Because the engineered yeast produces metabolites at the same level as a commercial yeast that is utilized in dozens of bioethanol plants currently and has been for over ten years, there is no increase in risk presented by the engineered yeast for an animal consuming DDG containing the engineered yeast.

6. Detailed summary of the basis for Notifier's GRAS determination

6.1 General safety assessment of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has an extensive history of use in food processing. Also known as Baker's Yeast or Brewer's Yeast, this organism has been used from as early as the Stone Age (about 9000 BC) as leavening for bread and as a fermenter of alcoholic beverages (Tucker, Woods 1995). But it was only between 1857 and 1863 that Louis Pasteur demonstrated the role played by yeasts, as the micro-organism responsible for fermentation. He noted at that time that one type of living cells was responsible for the fermentation of bread, beer, wine, cider corresponding to a population of a microscopic fungus, *Saccharomyces cerevisiae*. He discovered that a number of varieties of *Saccharomyces cerevisiae* exist in nature and are more or less adapted to the different fermentations (Pretorius 2000). *Saccharomyces cerevisiae* is considered Generally Recognized as Safe through its use in the brewing, baking and winemaking industry and is the subject of several GRAS Notices, 120, 175, 239, 260, 284, 350, 353 (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175, CFSAN / Office of Food Additive Safety 2008, GRN 000260, CFSAN / Office of Food Additive Safety 2008, GRN 000239, CFSAN / Office of Food Additive Safety 2009, GRN 000284, CFSAN / Office of Food Additive Safety 2010, GRN 000350, CFSAN / Office of Food Additive Safety 2010, GRN 000353).

Nowadays, the use of yeasts is not limited to the production of bread or fermented beverages. Since the 1930s yeast extract products were developed to enhance the flavor of a variety of products such as soups, sauces and ready-to-eat meals. Moreover, because of their nutritional characteristics (protein, vitamin, mineral and amino acid content), preparations of living cells or yeast cell walls have been commercialized as food supplements. Yeast is recommended for dietary supplementation for patients with diabetes type 2, diarrhea, high cholesterol and fatigue due to its high chromium and vitamin B content (see www.vitacost.com). In addition, the organism is widely used for the production of macromolecular cellular components such as lipids, proteins, enzymes, and vitamins (see www.vitacost.com; Moyad 2007, Moyad 2008).

Saccharomyces cerevisiae is a ubiquitous yeast. It is found on our bodies and in the air that we breathe, and it is naturally present on and in foods that man and animals eat regularly.

In its safety evaluation of carbohydrase from *Saccharomyces cerevisiae*, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered that *Saccharomyces cerevisiae* belongs to the group of micro-organisms traditionally accepted as constituents of foods or normally used in the preparation of foods (Joint FAO/WHO Expert Committee on Food Additives 1971).

In 1997 the US Environmental Protection Agency (EPA) issued their safety assessment of *Saccharomyces cerevisiae* and concluded that "*S. cerevisiae* is an organism which has an extensive history of safe use. Despite considerable use of the organism in research and the presence of *S. cerevisiae* in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the human had a debilitating condition. Tests for the factors associated with the virulence of yeasts (i.e., phospholipases) indicate that

this organism is nonpathogenic. The organism has not been shown to produce toxins to humans." (Environmental Protection Agency,1997) *Saccharomyces cerevisiae* was therefore included as a recipient microorganism at § 725.420 for the tiered exemption.

According to scientific experts, a nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism is defined as "one that is very unlikely to produce disease under ordinary circumstances." (Pariza, Foster 1983).

The European Food Safety Agency (EFSA) concluded that baker's yeast (*Saccharomyces cerevisiae*) fits this definition of nontoxigenicity and nonpathogenicity and that it is also not a major food allergen. EFSA therefore gave baker's yeast a Qualified Presumption of Safety (QPS) status (Opinion of the Scientific Committee, 2007). This means that in Europe, baker's yeast can be safely used in food and feed production and is exempt from the need for further safety assessment.

The FDA considers *Saccharomyces cerevisiae* and several derived products safe for consumption. Indeed, FDA has approved dried yeast as an ingredient for food (21 C.F.R. §172.896), and baker's yeast extract has been affirmed by the FDA as a GRAS flavoring agent and adjuvant (21 C.F.R. §184.1983). The FDA has also approved various yeast-derived products for their use in food. These include Baker's yeast protein (21 C.F.R. §172.325), Yeast-malt sprout extract (21 C.F.R. §172.590) and Baker's yeast glycan (21 C.F.R. §172.898).

In addition, GRAS notifications have been submitted and accepted by FDA with no questions for the use of genetically modified yeasts as starter cultures for wine (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175).

6.2 Safety of Inactivated Modified *Saccharomyces cerevisiae* for Production Animals

Because of the close relationship between humans and domestic animals and the practice of feeding domestic animals waste food products, animals have also been exposed to and consumed yeast for thousands of years. During World War II yeast preparations were recommended by the WHO as a protein substitute in animal nutrition. Presently, *Saccharomyces* supplements for animal nutrition can be found on the market. Yeast, yeast extracts and yeast cell wall fractions have been widely reported as safe and with positive influence on growth performance, meat quality and ileal mucosa development in poultry (Zhang, Lee et al. 2005, Owens, McCracken 2007). In the beef cattle industry, yeast-derived cell wall preparations are successfully used to minimize the toxic effects of high-ergot alkaloid tall fescue straw (Merrill, Bohnert et al. 2007). In pig feed, yeasts from *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are successfully replacing soy meal in order to avoid anti-nutritional factors from this type of meal (Spark, Paschertz et al. 2005). This work underlines the innocuousness of yeast and yeast extracts for animals in the different stages of life and in different species. In Europe, living *Saccharomyces cerevisiae* yeasts or extracts are permitted for use in animal nutrition in any animal species with no restriction on the amounts to be used (Dir 82/471/EEC). In the United States, supplements of *Saccharomyces cerevisiae* for animal

nutrition are commercially available for a variety of animals including cat, rabbit, rodents, birds, fish, dogs, goats, sheep, horses, cows and pigs. Amounts vary according to the size of the animals, but there is no limit in the duration of feeding (e.g. www.vi-cor.com, www.enzion.com). Section 96 of the American Association of Feed Control Officials 2013 Official Publication (Association of American Feed Control Officials 2013) lists nine definitions describing material containing or consisting solely of *Saccharomyces cerevisiae* or one or more of its components as being suitable for animal feed with no maximum levels noted other than good feeding practices. The earliest entry is from 1951 indicating that the state feed officials and CVM, due to their review of AAFCO listed materials, have considered *Saccharomyces cerevisiae* to be a safe and suitable feed ingredient for over 60 years.

6.3 Safety considerations due to the nature of modifications of *Saccharomyces cerevisiae*

The US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420). This exemption is permitted because genetically engineered strains of the species were found to have no adverse effects on man or the environment. They also determined that the introduction of intergeneric material will not increase the potential for adverse effects, provided that the genetic material is limited in size, well characterized, free of certain sequences and poorly mobilizable (Environmental Protection Agency, 1997).

For DSM's Modified *Saccharomyces cerevisiae*:

- the only additional heterologous genetic material was (b)(4) and the coding sequence of the inserted (b)(4) limited in size;
- the additional genetic material is completely characterized as it was made synthetically;
- the additional genetic material by default is free from sequences of concern, and the additional genetic material is poorly metabolizable as it is integrated in the genome of the host and lacks sequences which would allow it to be mobilized;
- no antibiotic resistance genes are present;
- no toxigenic genes were inserted;
- no antibiotic production genes were inserted.

The gene insertion was not random but targeted and the locations are known as illustrated in sections 2.3.5 and 2.4 of this dossier. The entire genome of *Saccharomyces cerevisiae* is known and it is free of toxin producing DNA sequences (see http://www.yeastgenome.org/ for the entire genome). It is not possible to induce the formation of a new attribute utilizing the techniques described in the organism construction sections of the dossier.

The Modified *Saccharomyces cerevisiae* that is the subject of this GRAS Notice is also the subject of the Microbial Commercial Activity Notice J13-0007 which was "dropped from review" by EPA in June 2013.

DSM also assessed the safety of the Inactivated Modified Yeast as it relates to its use in foods according to the decision tree guidelines developed by Pariza and Johnson (2001) for food enzymes and later modified by Pariza and Cook (2010) for animal feed enzymes derived from GE organisms. The test article was shown to be acceptable for feed use. In addition, the entire organism is boiled in acidic aqueous fermentation broth during the distillation step in biofuel production and destroyed as seen in Figure 2-13. The decision tree is in Annex 14.

6.4 Exposure for target animals

It is anticipated that the Inactivated Modified *Saccharomyces cerevisiae* will be utilized in animal feed either as an individual feed ingredient similar to traditional dried yeast or as a component of Distillers' Dried Grains with or without Solubles (DDG/S). As an individual feed ingredient the material will be added to the feed of poultry, swine, bovines, minor species and pets at levels not to exceed good manufacturing practices.

The table below presents the possible range of use and exposure of Modified *Saccharomyces cerevisiae*. It will be used alone or as a component of an ingredient (Dried Distillers' Grains) in the feed of the following production animals as part of a complete ration.

Table 6-1 Proposed use level and exposure of Production Animals to Modified *Saccharomyces cerevisiae*

Species /Class	Maximum Feed Consumption* (Kgs per day)	Maximum Level of Modified yeast in Feed from Table 3-1	Maximum intake of Modified yeast (Kg per day)	Number of days of consumption	Total exposure Kgs of yeast	Exposure (mg Modified yeast/kg BW/day+)
Broiler Chickens	0.11	2%	0.0022	48	0.106	1.22
Laying Hens	0.1	3%	0.003	365	1.09	1.67
Breeding Hens	0.1	3%	0.003	365	1.09	1.67
Piglet	0.2	5%	0.01	56	0.56	1.10
Sows	1.8	10%	0.18	365++	65.7	1.20
Lactating Sows	5.17	10%	0.517	579++	299.3	3.45
Finisher	4	4%	0.16	126	20.2	1.31
Turkeys	0.2	3%	0.006	140	0.84	0.63
Beef Cattle	10.6	5%	0.53	180	95.4	0.97

Species /Class	Maximum Feed Consumption* (Kgs per day)	Maximum Level of Modified yeast in Feed from Table 3-1	Maximum intake of Modified yeast (Kg per day)	Number of days of consumption	Total exposure Kgs of yeast	Exposure (mg Modified yeast/kg BW/day+)
Dairy Cows	22	5%	1.1	1460	1,606	2.09

* (Subcommittee on Feed Intake, Committee on Animal Nutrition, National Research Council 1987)
+ Average weight at slaughter
++ (Stalder, Engblom et al. 2009)

6.5 Information that may appear to be inconsistent with the GRAS determination

An extensive literature research on the safety of *Saccharomyces cerevisiae* has been performed by the European Food Safety Agency (Opinion of the Scientific Committee, 2007). The following is an extract of the EFSA report:

> *Saccharomyces cerevisiae (also known as "baker's yeast" or "brewer's yeast") is mostly considered to be an occasional digestive commensal. However, since the 1990's, there have been a growing number of reports about its implication as an aetiological agent of invasive infection in "fragile" populations. A particular feature of such infections is their association with a probiotic preparation of S. cerevisiae (subtype S. boulardii) for treatment of various diarrhoeal disorders (see below). The nature of S. cerevisiae (subtype S. boulardii) and its clinical applications are reviewed by Buts and Bernasconi* (Buts, Bernasconi 2005).
>
> *In one review, 92 cases of Saccharomyces invasive infection were presented* (Enache-Angoulvant, Hennequin 2005). *Predisposing factors were similar to those of invasive candidosis, with intravascular and antibiotic therapy being the most frequent. Blood was the most frequent site of isolation (78% or 72 patients). S. cerevisiae (subtype S. boulardii) accounted for 51.3% (47 cases) of fungaemias and was exclusively isolated from blood. Special caution should be taken regarding the use of S. cerevisiae (subtype S. boulardii) preparations* (Fleet & Balia 2006). *There are number of recent reports and reviews regarding the safety of S. cerevisiae (subtype S. boulardii) preparations involved in:*
>
> - *A case of Saccharomyces cerevisiae acquired fungaemia* (Cassone et al. 2003, Graf &Gavazzi 2007). *The authors concluded that probiotics should be used cautiously in certain high-risk populations.*
>
> - *A review of the current literature reinforces the view that fungaemia and sepsis are rare complications of the administration of S. cerevisiae (subtype S. boulardii) in immunocompromised patients but confirms that*

> *the most important risk factor for S. cerevisiae fungaemia is the use of probiotics* (Herbrecht, Nivoix 2005, Munoz, Bouza et al. 2005). *This raises the question of the risk-benefit ratio of these agents in critically ill or immunocompromised patients who are likely to develop an infection after exposure to high amounts of a microorganism with a low virulence.*
>
> *The body of knowledge is considered as sufficient (long history of safe use) with only 92 cases of pathogenic cases involving S. cerevisiae reported in total (15 cases diagnosed before 1990); all patients had at least one condition facilitating the opportunistic development of S. cerevisiae. S. bayanus and S. pastorianus are used in wine and beer production. There are no foodborne infection issues for these species.*

On the basis of their assessment, EFSA classified *Saccharomyces cerevisiae* as a QPS (Qualified Presumption as Safe) organism, a non-pathogen and not a major food allergen. *Saccharomyces* has been an approved component of feed since at least 1951 and there are no literature reports that identify *Saccharomyces cerevisiae* as toxic for animals. As stated earlier, this means that it can be safely used in food and feed without additional safety testing.

In addition, a comprehensive literature search was performed on April 15, 2013 by DSM Bio-based Products and Services. The scientific databases Medline, BIOSIS and EMBASE as provided by the host STN were searched simultaneously (see Annex 15). Usual terminology related to pathogenicity, toxicology or safety was used, and the following keywords were searched: *Saccharomyces cerevisiae*/Baker's yeast/Brewer's yeast,pathogen/tox/safe/allergen.

The search confirmed that although the cases of human infection with *Saccharomyces cerevisiae* are rare, some isolates have pathogenic potential. Some clinical isolates are notably known to be virulent in humans. In addition, several reports evaluating the safety of commercial *Saccharomyces cerevisiae* strains have clearly identified the subtype *boulardii* as a potential pathogen, in particular towards immunocompromised patients. See Cassone et. al., 2003 and Graf & Gavazzi, 2007)

Although very few scientific reports are available on potential adverse effects of *Saccharomyces cerevisiae* for animals, the few toxicity studies that were performed on rats, mice or monkeys with the yeast or with the yeast biomass do not suggest that these animals display a stronger sensitivity towards *Saccharomyces cerevisiae* than humans. (Caballero-Cordoba G.M. and Sgarbieri V.C. (2000), Byron J.K. et al., (1995), Maejima K. et al., (1980).

Recent efforts have focused on attempting to characterize the virulence traits related to *Saccharomyces cerevisiae,* thus enabling the means to better identify the potential pathogenic strains (de Lanos R. et al.,(2011) (McCusker J.H. et al., (1994)). The phenotypic traits of virulence of pathogenic strains are believed to be due to their ability to grow at high temperature (38-42^{0}C), their adherence and their ability to invade host cells, and their ability to produce and secrete degradative enzymes (i.e. proteinase and phospholipase) (de Lanos R. et al., (2011)). This search did not identify any reports of isolates of *Saccharomyces cerevisiae* able to produce toxins against animals or humans, although several studies have shown the ability of the yeast to produce toxins against other yeasts (the so-called killer toxins) (Orentaite et. al. (2012), Soares G.A.M and Sato H.H. (1999)).

In conclusion, we believe that this literature search does not alter the conclusions previously made by several bodies of experts, EFSA, EPA and FDA through their acceptance of several

GRAS Notices that *Saccharomyces cerevisiae* can be considered as a safe microorganism and a non-pathogen, although some of the yeast isolates should be regarded as opportunistic pathogens of low virulence.

6.5.1 Methyl Glyoxal

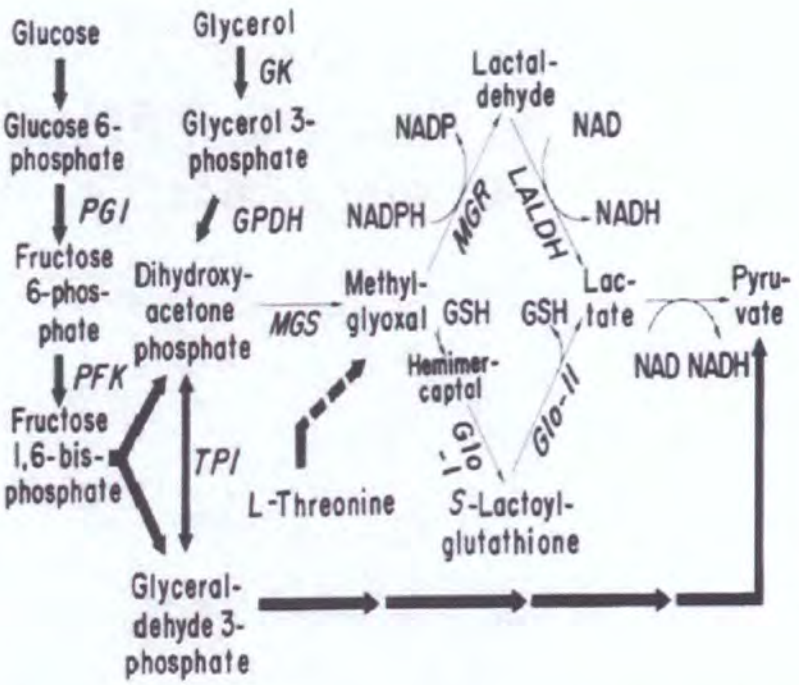
In metabolically active yeast cells, methylglyoxal may be formed during normal cell metabolism. Methylglyoxal synthase catalyzes the reaction from dihydroxyacetonephosphate to methylglyoxal. In 1997 Hashimoto et. al. found that genetically engineered *Saccharmoycese cerevisiae* strain DKD-5D-H produced and increased amount of methylglyoxal, a toxic 2-oxoaldehyde, compared to the wild-type yeast cells. This increase was reportedly due to the gentic manipulations involving the enzymes phosphoglucose isomerase, phosphofructokinase and triosephosphate isomerase.

In construct 1, overexpression of the *GLO1*-gene is brought about. To this end, the *Saccharomyces cerevisiae GLO1*-gene is expressed under control of the PGK1-promoter.

The *GLO1*-gene encodes monomeric glyoxalase I, which catalyzes the detoxification of methylglyoxal (a by-product of glycolysis) via condensation with glutathione to produce S-D-lactoylglutathione (see figure 4-5 below).

Figure 4-5 Methylglyoxal detoxification

Figure 1 Enzymic routes for synthesis and degradation of MG in *S. cerevisiae*. Non-enzymic formation of methylglyoxal (MG) from glycerol 3-phosphate and glyceraldehyde 3-phosphate is shown by Phillips & Thornalley (1993). The pathway indicated by thin lines represents pathways for MG degradation (glycolytic bypass). For abbreviations, see text.



The figure was copied from Hashimoto et. al. (1997) International Journal of Food Science and Technology **32**, 521-526.

In section 2.5.2 it is noted that overexpression of the GLO1-gene helps in lowering the intracellular glyoxal levels.

6.6 Summary

Saccharomyces cerevisiae is ubiquitous, with man and animals being exposed to the organism on a regular basis through exposure to air, water, soil and food. The organism is composed of primarily proteins, nucleotides, and complex carbohydrates and has not been reported to be toxic nor allergenic for animals. Yeast is utilized as a component of animal food for its nutritional composition, the Modified *Saccharomyces cerevisiae* that is the subject of this notice is not compositionally different from traditional yeast fed to animals as noted in section 2.20 of this dossier. A detailed analysis of the amino acid profile did not reveal any statistically significant differences between the DSM Modified *Saccharomyces cerevisiae* and traditional yeast used for commercial ethanol production from corn.

Saccharomyces cerevisiae has been widely and safely fed to poultry, cattle and other livestock due to its nutritious properties for over 60 years as evidenced by the ingredient definitions in the AAFCO Official Publication. A variety of *Saccharomyces* ingredients for animal nutrition can be found on the market. Yeast, yeast extracts and yeast cell wall fractions have been widely reported as safe and with positive influence on growth performance, meat quality and ileal mucosa development in poultry (Zhang et al., 2005; Owens and McCracken, 2007).

The safety of *Saccharomyces cerevisiae* is recognized by FDA, EPA and EFSA; and is confirmed by the literature. FDA considers *Saccharomyces cerevisiae* and several derived products as GRAS and therefore safe for consumption.

Although there have been reports of infection by *Saccharomyces cerevisiae* in immune-compromised individuals, it is not considered to be pathogenic.

The entire genome of the organism is known and is devoid of known toxigenic sequences. The organism that is the subject of this GRAS Notice was modified following NIH guidelines and has been accepted by the EPA as safe for large scale industrial fermentation and use for the production of bioethanol. The modifications to the organism only increased the number of native genes already present and added one gene for the production of an enzyme that is currently approved for use in food production for both animals and humans. The ability to produce novel amino acids or proteins was not introduced. The gene coding for the non-native enzyme was synthetic and therefore devoid of any extraneous DNA from the template organism. Therefore the Modified *Saccharomyces cerevisiae* does not possess unusual genetic material nor the ability to produce unusual toxic substances that would make its toxicological properties different than that of traditional yeast fed to animals.

It is anticipated that the exposure of target animals to the Genetically Engineered, Inactivated Modified *Saccharomyces cerevisiae* will not be higher than the current exposure to classical *Saccharomyces cerevisiae*. Yeast is already a component of DDG with or without solubles and the DSM yeast is a one for one replacement of the currently used microorganism.

In August, 2013, at their annual meeting, AAFCO accepted the petition of the Mascoma Corporation and formed a new ingredient definition for a genetically engineered *Saccharomyces cerevisiae* developed for bioethanol production from corn that contained four genes from two

donor species, a yeast and a bacterium. In their review, CVM noted that the purpose of the 'yeast is to produce fuel-ethanol from dry grind corn and that 'The distillers products will be used in animal food, but will not contain viable bioengineered yeast.'(see CVM, 2013). Therefore, CVM has previously reviewed a genetically engineered yeast used for bioethanol production from a similar substrate and found that the inactivated yeast was suitable for use in a similar feed ingredient, distillers' grains.

Conclusion:

The genetically engineered pentose fermenting *Saccharomyces cerevisae*, refered to in this document as Modified *Saccharomyces cerevisiae*, produced during bio-fuel production from food grade plant- based materials such as corn, wheat and sorghum is safe for consumption by production animals, minor species and pets. The Modified *Saccharomyces cerevisiae* is substantially equivalent to the traditional yeast utilized as an ingredient in animal food.

7. Human safety

Human exposure is anticipated to be limited to individuals handling the material during packaging at the yeast production facility, the bio-ethanol facility, in the manufacture of feed, on the farm, and from consumption of the target animals. The use of personal protective equipment and compliance with normal occupational safety practices will minimize exposure.

To our knowledge, there are no toxicokinetic studies on *Saccharomyces cerevisiae*. However, inactivated Modified *Saccharomyces cerevisiae* will be metabolized during animal digestion into essential compounds consisting mainly of proteins and carbohydrates. Amino acids and peptides are important building blocks in the human body. Carbohydrates are indispensable for storage or transport of energy, and they also play a role in immune function, blood clotting and development.

Saccharomyces cerevisiae is in general considered to be of very low toxicity for humans. This is based on human evidence coming from a long history of safe use, on its composition and on the fact that it is readily biodegradable in the human gastro-intestinal tract. In the public domain, toxicity studies on *Saccharomyces cerevisiae* are limited. However, due to low risk expected for man and the environment, the US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism is exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420).

7.1 Allergenicity

It is well known that when inhaled, proteins in general can cause sensitization and allergic reactions. Inhalation allergy towards *Saccharomyces cerevisiae* has been described by Baldo and Baker (Baldo, Baker 1988). This type of allergy is predominantly observed in bakers.

Regarding oral allergy from ingestion of *Saccharomyces cerevisiae,* only one possible case is known (Pajno, Passalacqua et al. 2005). An atopic 6-year old boy experienced generalized urticaria and asthma after eating pizza and bread fresh from the oven (within one hour). The reaction towards yeast was confirmed in a skin prick test procedure. The reaction only occurred when the bread just had been prepared, whilst no symptoms occurred when the bread was eaten more than one hour after preparation. Since this phenomenon is uncommon with regard to allergens, the cause of the boy's allergic reaction remains inconclusive. In its assessment of *Saccharomyces cerevisiae* for a classification as QPS (Qualified Presumption as Safe) organism, the European Food Safety Agency (EFSA) concluded that *Saccharomyces cerevisiae* is not a major food allergen (Opinion of the Scientific Committee, 2007).

In addition, the impact of the modifications made on the *Saccharomyces cerevisiae* portion of DDGS was evaluated by applying the guidelines on testing the potential allergenicity of genetically modified foods as presented by the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (Food and Agriculture Organization of the United Nations/World Health Organization 2001, Food and Agriculture Organization of the United Nations/World Health Organization 2009). According to the guidelines cross-reactivity between the expressed protein and a known allergen has to be considered when there is:

1) more than 35% identity in the amino acids sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty

or

2) identity of short contiguous amino acids segments (i.e. at least 8 contiguous amino acids). Although the 2001 WHO/FAO consultation suggested searching for matches of 6 identical amino acid segments or longer, it is recognized that a search for such small sequences would lead to the identification of too many false positives (Food and Agriculture Organization of the United Nations/World Health Organization 2009). It has been reported that an immunologically significant sequence similarity requires a match of at least 8 contiguous identical residues (Fuchs, Astwood 1996, Metcalfe, Astwood et al. 1996).

The Inactivated Modified *Saccharomyces cerevisiae* has been engineered to (b)(4) several *Saccharomyces cerevisiae* genes as well as (b)(4) from another yeast species, but no foreign DNA sequences known to code for allergenic proteins were inserted into the organism.

Because (b)(4) not native to *S. cerevisiae*, and *S. cerevisiae* is of low allergenic impact for humans only the foreign gene was extensively evaluated.

For comparison, DSM used the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated February 12, 2013). The comparison was done in March 2013. AllergenOnline™ allows the search in NCBI, SwissProt, PIR, PRF, PDB and the WHO-IUIS databases using a FASTA algorithm. The WHO-IUIS list is set up by the IUIS Allergen Nomenclature Sub-committee operating under the auspices of the International Union of Immunological Societies and the World Health Organization. The objectives of the IUIS Allergen Nomenclature Sub-committee are to maintain a unique and unambiguous nomenclature for allergen molecules and maintain the 'official list of allergens'.

In addition, the amino acid sequence comparison of the inserted heterologous gene protein product (see Annex 16) did not show 35% or more overlap with known allergens using a window of 80 amino acids. Exact matches of 8 amino acids or more were not observed.

Therefore, it is concluded that the inserted gene protein product has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

A material safety datasheet with additional information on the Modified*Saccharomyces cerevisiae* is provided in Annex 17.

8. Environmental Safety

Saccharomyces cerevisae is exempted from TSCA Section 5 PreMarket Notification by the Environmental Protection Agency (40 CFR §725.420, the Environmental Protection Agency 1997). The EPA has reviewed and approved the use of this genetically engineered *Saccharomyces cerevisiae* for biofuel production under Microbial Commercial Activity Notice (MCAN) J11-0001. Another genetically engineered yeast, *Pichia pastoris*, has also been approved under MCAN J04-0003. DSM has filed a Tier 1 exemption notice with the EPA which was accepted by the agency in December 2011 and a Microbial Commercial Acitvity Notice which was accepted on 18 March 2013 and dropped from review on 12 June 2013.

Inactivated Modified *Saccharomyces cerevisiae* is a GRAS substance and per 21 CFR §25.32 (k), foods, food additives and color additives, including GRAS substances, are categorically excluded from the requirement to provide an environmental impact statement or an environmental assesment. Inactivated Modified *Saccharomyces cerevisiae* will be added directly to animal food and is intended to remain in the food through ingestion by animals and it is not intended to replace macronutrients in animal food.

There is no information on the ecotoxicity of Modified *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* in the public domain. However, because of the macro-composition of Modified *Saccharomyces cerevisiae*, consisting mainly of proteins and carbohydrates, it can be expected that the substance is readily biodegradable. Thefore, no toxicity for invertebrates, aquatic plants and fish is expected, since the substance will be degraded by bacteria in the water. In addition, as was previously indicated, *Saccharomyces cerevisiae* has been fed widely to poultry, cattle and other livestock due to its nutritious properties and is thus of very low toxicity. It is also used as nutritious mixture in *in vivo* ecotoxicity tests, meaning that many animals in ecotoxicity studies are fed or supplemented with *Saccharomyces cerevisiae*.

9. Annexes

1 Stability of the inserted genetic material
2 Taxonomy of the donor
3 Confirmation of growth on xylose
4 Antibiotic resistance
5 (b)(4)
6 Inactivation study
7 Laboratory method for inactivation study
8 CoA for *Saccharomyces cerevisiae*
9 Modified*Saccharomyces cerevisiae* nutritional analysis
10 Excerpt of Pilot Plant Report
11 Ethanol Fermentation Raw Material Specifications
12 DDG Amino Acid Analysis Statistics
13 DDG Digestability Statistics
14 Pariza & Cook Decision Tree
15 Literature search
16 Allergen Search
17 MSDS Advanced *Saccharomyces cerevisiae*
18 Yeast Fermentation Raw Material Specifications
19 Stability of yeast fermentation performance
20 Spill-over experiment report

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Section 172: FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION

§ 172.325 - Bakers yeast protein.

§ 172.590 - Yeast-malt sprout extract.

§ 172.896 - Dried yeasts.

§ 172.898 - Bakers yeast glycan.

Section 184: DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

§ 184.1983 - Bakers yeast extract.

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12. Expert Panel Consensus Statement Concerning the Determination of the Safety and Generally Recognized as Safe ("GRAS") Status of the Proposed Use of a Genetically Modified Pentose Fermenting *Saccharomyces Cerevisiae* as a Component of Animal Feed

November 26, 2013

INTRODUCTION

At the request of DSM Nutritional Products, (DSM), a panel (the "Expert Panel") of independent scientists, qualified by scientific training and relevant national and international experience to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information on technical information that supports the Generally Recognized as Safe (GRAS) status of an Inactivated Modified *Saccharomyces cerevisiae* as a nutritional product for use in poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets, based on scientific procedures. The Inactivated Modified *Saccharomyces cerevisiae* will be present in dry distillers' grains with or without solubles at up to 20% of the dry solids. For purposes of this evaluation, "safe" or "safety" as it relates to GRAS within the terms of the Federal Food, Drug, and Cosmetic Act means that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2012).

The organism that is the subject of this GRAS assessment was modified following NIH guidelines and has been accepted by the EPA as safe for large scale industrial fermentation and use for the production of bioethanol. The Modified *Saccharomyces cerevisiae* is derived from the same genus and species as baker's yeast. The nutritional composition of Modified *Saccharomyces cerevisiae* is therefore expected to be very comparable to that of dry baker's yeast already on the market. This was confirmed by comparing the nutritional composition of three lots of Modified *Saccharomyces cerevisiae* with the composition of active dry baker's yeast obtained from the USDA National Nutrient Database (USDA 2013), and commercialized baker's yeast. The Modified *Saccharomyces cerevisiae* is composed of proteins, lipids, carbohydrates, cellulose and minerals. The commercial product for bioethanol production will be sold as either a dry powder produced from a concentrated liquid or the concentrated liquid itself (commercially referred to as 'cream yeast') by DSM Bio-based Products & Services. The inactivated yeast will be a byproduct following distillation of ethanol and a component of Dried Distillers' Grains with or without Solubles (DDG/S) or sold alone as dry yeast.

The modifications to the organism only increased the number of native genes already present and added (b)(4), that is currently

approved for use in food production for both animals and humans. The ability to produce novel amino acids or proteins was not introduced. The gene coding for the non-native enzyme was synthetic and therefore devoid of any extraneous DNA from the template organism.

It is anticipated that the exposure of target animals to the Genetically Engineered, Inactivated Modified *Saccharomyces cerevisiae* will not be higher than the current exposure to classical *Saccharomyces cerevisiae.*

The Expert Panel consisted of the below-signed qualified scientific experts: Mark E. Cook, Ph.D., (University of Wisconsin - Madison), John A. Thomas, Ph.D, F.A.T.S (Indiana University School of Medicine) and Stanley M. Tarka, Jr. Ph.D. (Chair) (The Tarka Group, Inc. and The Pennsylvania State University). Copies of *curricula vitae* evidencing the qualifications of the Expert Panel members are attached.

The Expert Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other sources through November 10, 2013 by DSM Bio-based Products & Services and DSM Nutritional Products and other information deemed appropriate or necessary. The Expert Panel evaluated information on characterization of the source yeast and the production strain of *Saccharomyces cerevisiae* under consideration, manufacturing/production methods, compositional data, specifications and batch analyses, intended use-levels, consumption estimates for the intended uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of this inactivated Modified *Saccharomyces cerevisiae* as compared to classical *Saccharomyces cerevisiae.*

The Expert Panel convened via teleconference and unanimously concluded that for the intended uses of Inactivated Modified *Saccharomyces cerevisiae* produced from a genetically engineered *Saccharomyces cerevisiae*, meeting appropriate food-grade specifications as described in the supporting dossier and manufactured consistent with current Good Manufacturing Practice (cGMP), is GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

SUMMARY OF THE BASIS FOR GRAS STATUS OF THE INTENDED USES OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED

DSM Nutritional Products provided technical information that supports the Generally Recognized as Safe (GRAS) status of Inactivated Modified *Saccharomyces cerevisiae* (also known and commercially marketed as Inactivated "Advanced *Saccharomyces cerevisiae*"), a nutritional product for use in poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets, based on scientific procedures.

DSM Bio-based Products and Services (DSM-BPS) developed this modified yeast; their sister company, DSM Nutritional Products will manufacture and package the product. DSM-BPS will market the product. Modified *Saccharomyces cerevisiae* is used by bio-ethanol facilities for the fermentation of sugars released from plant-based feed stocks, such as corn, into ethanol in a manner similar to the production of beer or grain neutral spirits used for in food and pharmaceutical processing. Inactivated Modified *Saccharomyces cerevisiae* is a heat killed co-product of bio-ethanol production.

Although the yeast was developed for use in bio-ethanol production, DSM-BPS is aware that potential customers may separate the inactivated yeast from the spent stillage for sale as an ingredient for animal feed or it will be dried with the cellulosic material in the stillage and become part of the Wet or Dried Distillers Grains with or without solubles (DDG/S and DDG) depending upon the business practices of the potential customer. These practices are commonly followed today with conventional *Saccharomyces cerevisiae* and the use of the yeast and DDG/S are permitted under feed ingredient definitions of the American Association of Feed Control Officials (AAFCO) as overseen by FDA's Center for Veterinary Medicine, at levels not exceeding good manufacturing procedures (AAFCO O.P. Sections 96 and 27 respectively). DSM-BPS does not intend to exceed the current limits for use of yeast and merely seeks to expand its permitted use to include this genetically engineered strain in an inactivated format.

The subject yeast will be produced in two product forms: a concentrated 'cream yeast' and a dry form commonly referred to as 'active dry yeast'. It is anticipated that the dry form will be the predominant marketed form for bio-ethanol production. Initially the Modified *Saccharomyces cerevisiae* is produced by (b)(4) in a facility that follows food cGMP (21 CFR§ 110). The yeast is then used in bio-ethanol production where it multiplies several times during the fermentation cycle. The yeast is them inactivated during the distillation process. Since the host organism is safe, the incorporated DNA does not encode any known harmful or toxic substances, and the active yeast is inactivated during the distillation process, the resulting inactivated genetically engineered organism is considered to be safe.

Saccharomyces cerevisiae is a ubiquitous microorganism found all over the globe and has been utilized by humans serendipitously and intentionally for the production of bread, beer and wine for over six thousand years. The National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules (Department of Health and Human Services 1986) considers *Saccharomyces cerevisiae* a safe organism.

The safety of the Inactivated Modified *Saccharomyces cerevisiae* has been established based on the published literature and history of use. A comprehensive search of the scientific literature for safety and toxicity information on *Saccharomyces cerevisiae* was conducted by DSM Bio-based Products and Services. The databases searched included Medline, BIOSIS and EMBASE databases as provided by the host STN.

The basis for the GRAS determination of the intended uses of Inactivated Modified *Saccharomyces cerevisiae* produced by (b)(4) in a facility that follows food cGMP from an original *Saccharomyces cerevisiae* unmodified parent strain (b)(4) (classical wild-type isolate) as a nutrient is through scientific procedures. Additionally, (1) pathogenicity of the taxonomic designation is rare and a outcome of other medical complications, (2) all the modifications are under the genetic control of a *Saccharomyces cerevisiae* promoter and terminator and are integrated in the genome of the yeast, (3) all additional gene sequences have been constructed *in-vitro*, based on the information on the genes as available in gene and/or protein databases, (4) the risk that DNA from the donor organism coding for antibiotic resistance was transferred to the recipient host strain is non-existent, (5) bacterial vectors used in the construction and the selection marker genes have been removed from the final strain (6) it is anticipated that the exposure of target animals to the Genetically Engineered, Inactivated Modified *Saccharomyces cerevisiae* is nonexistent since the organism is killed during the distillation process (7) extensive history of safe use and consumption by animals and humans of inactivated *Saccharomyces cerevisiae* and (8) safety is recognized by multiple regulatory authorities of the parent lineage (FDA, EPA, EFSA). The primary products produced by fermentations utilizing the engineered yeast are yeast biomass and ethanol and in that respect the engineered yeast is not different from the conventional yeast normally used for alcoholic fermentations when inactivated. The Inactivated Modified *Saccharomyces cerevisiae* will be sold either as a single feed ingredient or become part of Distillers Dried Grains with or without Solubles and is intended to be used as a nutrient source. It is substantially equivalent to the host strain, common baker's yeast, from which it is derived, and is therefore Generally Recognized As Safe for use as an ingredient in animal food.

The compositional equivalence of Inactivated Modified *Saccharomyces cerevisiae* is shown by the nutritional analysis of two pilot plant batches and a sub-sample of one commercial batch of "Advanced *Saccharomyces cerevisiae*" as compared to the published nutritional data of active dry baker's yeast and to the nutritional analysis of a sample of commercial baker's yeast. As anticipated, because the "Advanced *Saccharomyces cerevisiae*" is the same in basic molecular composition as conventional yeast, there was no significant difference between the different yeast products.

All relevant information was reviewed, summarized and incorporated into a GRAS dossier, 'THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED' and submitted to the GRAS Panel. The GRAS Panel received information pertaining to the method of manufacture, product specification, analytical data, intended use levels in animal food for avian, ruminant and porcine species, resulting consumption estimates from the intended uses, and any

other relevant data on safety and tolerance-related information. The members of the Expert Panel were Mark Cook, PhD, John A. Thomas, PhD, Fellow, ATS, and Stanley M. Tarka, Jr., PhD (Panel Chair).

SAFETY OF MODIFICATIONS INTRODUCED INTO THE ORGANISM

The original *Saccharomyces cerevisiae* parental strain is also known as baker's yeast or brewer's yeast. The unmodified (b)(4) was identified in the laboratory by (b)(4) and was deposited at (b)(4) lists the unmodified source organism as *Saccharomyces cerevisiae* (b)(4). Subsequently, (b)(4) was obtained by and modified at (b)(4) . The resulting modified strain was designated as (b)(4) .

The nature of the unmodified parent strain, (b)(4), is that it is a congenic member of the (b)(4) family as described by (b)(4). The parent strain, (b)(4) was a classical wild-type isolate.

There are no known pathogens associated with this strain of *Saccharomyces cerevisiae* , thus, the designation is sufficient to distinguish the organism from species that exhibit pathogenicity under the genus *Candida.*

- *Saccharomyces cerevisiae* has been modified to (b)(4) (b)(4)

All the modifications are under the genetic control of a *Saccharomyces cerevisiae* promoter and terminator and are integrated in the genome of the yeast.

All additional gene sequences have been constructed *in-vitro*, based on the information on the genes as available in gene and/or protein databases. The risk that DNA from the donor organism coding for antibiotic resistance was transferred to the recipient host strain is non-existent.

Bacterial vectors used in the construction and the selection marker genes have been removed from the final strain.

The (b)(4) genes enable the yeast to metabolize C5 sugars in addition to the normal C6 sugars, and thus produce ethanol from (b)(4) feedstocks.

Nevertheless, the primary products produced by fermentations utilizing the engineered yeast are yeast biomass and ethanol and in that respect our engineered yeast is not different from the conventional yeast normally used for alcoholic fermentations.

PRODUCTION STRAIN

The strain phenotype is understood to refer to the expression of the genes of the organisms as well as the influence of environmental factors and random variation.

The unique characteristic that differentiates (b)(4) is its ability to efficiently ferment xylose in plant hydrolysates containing hemicellulose derived pentoses and acetic acid. To obtain this, the strain has an (b)(4) . That is what distinguishes the strain from other strains.

The strain phenotype is (b)(4)

Regarding phenotypic changes resulting from modifications to the microorganism, all gene sequences, except for (b)(4) , were sourced from the host strain *Saccharomyces cerevisiae*. As previously described, (b)(4) into ethanol.

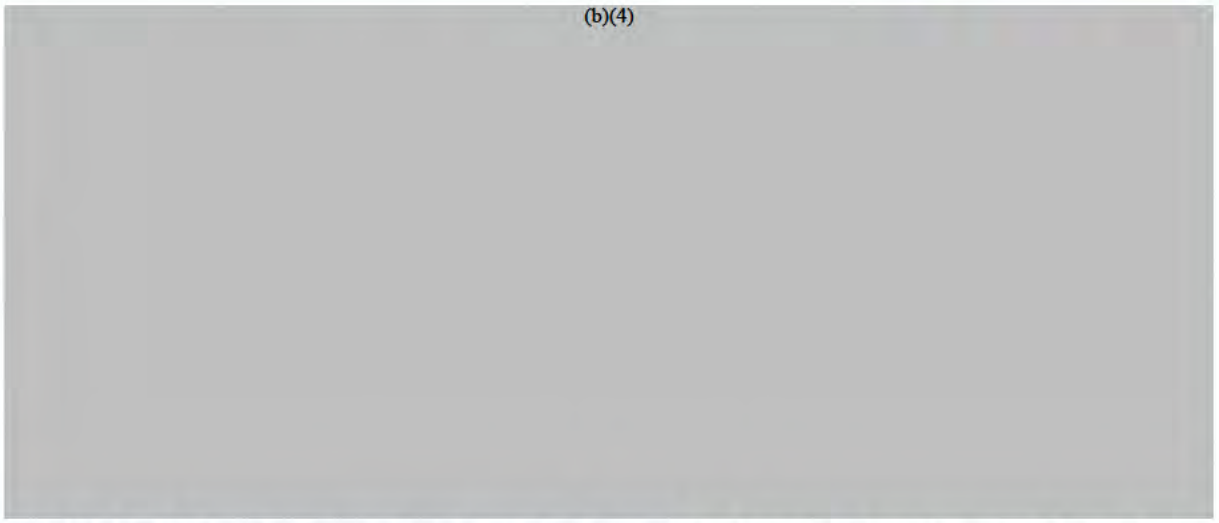
MANUFACTURING

Modified *Saccharomyces cerevisiae* is manufactured by DSM Nutritional Products following Good Large Scale Fermentation Practices and is in compliance with the applicable sections of 40 CFR §725. Inactivated Modified *Saccharomyces cerevisiae* is a co-product of the manufacture of the bio-fuel industry. Inactivated Modified *Saccharomyces cerevisiae* will enter the animal food chain as a by-product of ethanol production from grains, such as corn, wheat, barley, rye and other forage materials. It is anticipated that the substance will be isolated from the stillage of the Bio-fuel plant and sold a nutritive ingredient or it will be a component of the dried stillage commonly referred to as 'dried distillers' grains' (DDG). The yeast and the enzymes it expressed are inactive in the feed ingredient.

The Modified *Saccharomyces cerevisiae* was developed for the purpose of providing a robust yeast capable of metabolizing C5 and C6 sugars that could be easily added to the fermentation tanks of fuel ethanol producers. To accomplish this goal, the modified organism is produced in large quantities and packaged in suitable containers in a manner similar to standard baker's yeast as illustrated in the following diagrams. The organism is grown in a (b)(4) process; (b)(4) .

(b)(4)

(b)(4)



The Modified *Saccharomyces cerevisiae* is designed for the optimal production of bio-ethanol. The inactivated yeast is recovered from the stillage of the bio-fuel plant along with the unfermented plant matter in the same manner as traditional fermentation processes.

Under the current processing methods followed in a fuel ethanol facility, the Modified *Saccharomyces cerevisiae* will be exposed to lethal conditions in the distillation process step ensuring that only non-viable cells are incorporated into the feed of animals. The temperature and exposure time in the distillation step are above the generally accepted maximum for enzyme stability. The added enzymes were not selected for temperature stability and to the knowledge of DSM are not thermo-stabile. Data provided in the GRAS dossier contains the results of experiments that confirm that the organism is destroyed when exposed to normal bio-fuel distillation conditions. The references 'Alcohol Textook, 'chapters 16 and 19 (Richards 2009, Earnest, Snyder et al. 2009) provided additional information regarding fermentation plant operations that support the premise that the yeast will be killed during downstream processing at the biofuel plant.

COMPOSITION

The Modified *Saccharomyces cerevisiae* is composed of proteins, lipids, carbohydrates, cellulose and minerals. The commercial product for bioethanol production will be sold as either a dry powder produced from a concentrated liquid or the concentrated liquid itself (commercially referred to as 'cream yeast') by DSM Bio-based Products & Services. The inactivated yeast will be a component of Dried Distillers' Grains with or without Solubles (DDG/S) or sold alone as dry yeast.

Certificates of analysis for typical Modified *Saccharomyces cerevisiae* batches demonstrate consistency of production lots to established specifications (Table 1).

Table 1 Modified *Saccharomyces cerevisiae* liquid concentrate specifications and batch to batch variation

Parameter	Units	Specification (cream yeast)	Pilot Batch Rpt 1202 CRY	Batch C177000	Batch C177001
Appearance		Turbid cream yeast (concentrated broth) with a white/beige color	complies	complies	complies
Dry matter	%	(b)(4)			
pH					
Glucose	g/l				
Microbial contamination: total bacterial count	CFU/mL				
Microscopic observation in the EOF broth		conform	complies	complies	complies

The Modified *Saccharomyces cerevisiae* is derived from the same genus and species as baker's yeast. The nutritional composition of Modified *Saccharomyces cerevisiae* is therefore expected to be very comparable to that of dry baker's yeast already on the market. This was confirmed by comparing the nutritional composition of three lots of Modified *Saccharomyces cerevisiae* with the composition of active dry baker's yeast obtained from the USDA National Nutrient Database (USDA 2013), and the commercialized baker's yeast. Any difference in fiber content is due to methods of expressing fiber. In the active dry baker's yeast reported on the USDA database, the total dietary fiber content was determined, whereas only crude fiber content was measured in Modified *Saccharomyces cerevisiae* and a commercial baker's yeast (Table 2).

Table 2 Nutritional properties of the Modified *Saccharomyces cerevisiae*

Parameter	Units	Leavening agent, active dry baker's yeast*	Lot 1	Lot 2	Lot3	Baker's yeast (b)(4)
			1315 A	K160 A	1313 EF	
Dry Matter	%	94.9	92.2	92.1	89.7	95.9
Energy	kcal/Kg	3250	3218	3253	3145	3405
Protein	%	40.44	46.10	46.15	46.01	44.09
Fat	%	7.61	6.16	6.09	5.77	4.17
Crude Fiber	%	26.9**	<0.2	<0.2	<0.2	<0.2
Ash	%	NA	9.06	7.85	8.10	4.57
Ca	%	0.03	0.005	<0.004	0.004	0.09
P	%	0.637	1.88	1.54	1.33	0.79
K	%	0.955	2.26	2.54	2.16	1.55
Mg	%	0.054	0.27	0.18	0.19	0.09
Zn	ppm	79	121	106	93	80
Mn	ppm	NA	8	7	11	5
Cu	ppm	NA	7	9	12	2
Fe	ppm	22	49	11	68	54

NA: not available

* USDA National Nutrient Database for Standard Reference Release 26

** Total dietary fiber

Ash content is driven by the media composition. Baker's yeast has been commercially produced for over 75 years and the media composition has been optimized to be the least expensive formulation that allows for maximum cell mass in the shortest period of time. The media formulation for the Modified *Saccharomyces cerevisiae* has not been optimized at this point in time and may be too rich in minerals. DSM anticipates that the concentration of the media components may be altered in the future.

The difference in protein is driven by differences in the parent strain, media composition and the differences in the fermentation process. The Modified *Saccharomyces cerevisiae* has been developed to efficiently perform in biofuel substrates such as crude corn digests. Baker's and brewer's yeast used in feed are valued for their protein content and an increase in protein would be welcomed by the feed industry.

High protein levels (up to 60% on a dry matter (dm) basis is possible) lead to high yeast activity in bread applications, however they lead to a lower shelf life of the (fresh) yeast. In the case of dry yeast, an additional factor is that high protein yeast will not sufficiently survive the drying process. The yeast protein in baker's yeast products may vary from 40% to 60-65% on dm. For active dry yeast it may vary from 40-55% on dry matter (Reed, Peppler 1973).

The specification in the 2013 AAFCO OP definition 96.1 for dried yeast (Association of American Feed Control Officials 2013) is that the organism is *Saccharomyces cerevisiae* and has a minimum protein content of 40%. The Modified *Saccharomyces cerevisiae* that is the subject of this GRAS assessment easily meets this requirement.

Three batches of Dried Distillers' Grains with Solubles (DDG/S) containing the Inactivated Modified *Saccharomyces cerevisiae* were produced via fermentation of corn in a commercial bio-fuel processor following their normal process. The use of the Modified *Saccharomyces cerevisiae* in a bioethanol fermentation did not alter the specifications of the resulting dried distillers' grains with solubles (DDG/S). Although the dry yeast itself currently has an elevated protein and ash content compared to traditional baker's yeast, those differences do not alter the composition of the commercial feed ingredient in a significant manner (Table 3).

Table 3 Physical properties of the Dried Distillers' Grains with Solubles containing the Modified *Saccharomyces cerevisiae*

Parameter	Units	Specification	Lot 1 27-510	Lot 2 27-506	Lot3 27-502
Physical state		granules	granules	granules	granules
Solubility		complete and clear	complete and clear	complete and clear	complete and clear
Protein	%	30-35	32.69	33.68	31.69
Loss on drying	%	max 6	5.12	4.89	3.81
Residue on ignition	%	max 5	3.92	3.72	3.22
carbohydrates	%	max 50	44.5	46.1	44.4

These data collectively support the use of Inactivated Modified *Saccharomyces cerevisiae* as a suitable nutritional ingredient for animal feed.

Stability

The Modified *Saccharomyces cerevisiae* has been developed for use in the fermentation of biomass-derived carbohydrates for the purpose of producing fuel ethanol. The organism is not designed for nor is it expected to be stored for long periods of time before use in the biofuel plant. However, a stability study has demonstrated that the Inactivated Modified *Saccharomyces cerevisiae* retains its fermentative capacity after 6 months at 4° C, the recommended storage temperature.

INTENDED USE, USE LEVEL AND EXPOSURE

It is not expected that the amount of Inactivated Modified *Saccharomyces cerevisiae* in DDG/S or feed will be higher than the amount of classical *Saccharomyces cerevisiae* already found in feed or DDG/S, as a byproduct of conventional ethanol distillation. It was estimated that dried yeast represent 20% by weight of the dried distillers' solubles (Liu 2011).

The expected animal intake of DDG/S containing Inactivated Modified *Saccharomyces cerevisiae* is equal or less than the current intake of DDG/S containing classical *Saccharomyces cerevisiae* derived from starch-based biomass. These intakes depend on the nutritional needs of the specific target animals and are summarized in the table below (Table 4).

Since the nutritional content of Inactivated Modified *Saccharomyces cerevisiae* is very similar to classical *Saccharomyces cerevisiae* (see Table 2), the intake levels of Inactivated Modified *Saccharomyces cerevisiae* by the target animals will not exceed what is expected from classical *Saccharomyces cerevisiae*.

The following table (Table 4) illustrates the maximum percentage of modified yeast in the diet based upon the literature for DDG/S in the diet and the quantity of yeast in DDG/S (Liu 2011).

Table 4 Intake levels of DDG/S in feed of target animals

Species / Class	DDG/S Maximum % of Diet	Modified *Saccharomyces cerevisiae* Maximum % of Diet
Swine (Shurson, Spiehs et al.)		
Nursery pigs (>15 lbs)	25	5
Grow-finish pigs	20	4
Developing gilts	20	4
Gestating sows	50	10
Lactating sows	20	4
Boars	50	10
Poultry (National Corn Growers Association 2008)		
Chicken - Broilers	10	2
Chicken – Layers	15	3
Turkeys (grow/finish)	15	3
Bovines (Weiss, Eastridge et al.)		
Beef cattle	25	5
Dairy cows	25	5

It is anticipated that the Inactivated Modified *Saccharomyces cerevisiae* will be utilized in animal feed either as an individual feed ingredient similar to traditional dried yeast or as a component of Distillers' Dried Grains with or without Solubles (DDG/S). As an individual feed ingredient the material will be added to the feed of poultry, swine, bovines, minor species and pets at levels not to exceed good manufacturing practices.

The table below (Table 5) presents the possible range of use and exposure of Modified *Saccharomyces cerevisiae*. It will be used alone or as a component of an ingredient (Dried Distillers' Grains) in the feed of the following production animals as part of a complete ration.

Table 5 Proposed use level and exposure of Production Animals to Modified *Saccharomyces cerevisiae*

Species /Class	Maximum Feed Consumption* (Kgs per day)	Maximum Level of Modified yeast in Feed from Table 4	Maximum intake of Modified yeast (Kg per day)	Number of days of consumption	Total exposure Kgs of yeast	Exposure (mg Modified yeast/kg BW/day+)
Broiler Chickens	0.11	2%	0.0022	48	0.106	1.22
Laying Hens	0.1	3%	0.003	365	1.09	1.67
Breeding Hens	0.1	3%	0.003	365	1.09	1.67
Piglet	0.2	5%	0.01	56	0.56	1.10
Sows	1.8	10%	0.18	365++	65.7	1.20
Lactating Sows	5.17	10%	0.517	579++	299.3	3.45
Finisher	4	4%	0.16	126	20.2	1.31
Turkeys	0.2	3%	0.006	140	0.84	0.63
Beef Cattle	10.6	5%	0.53	180	95.4	0.97
Dairy Cows	22	5%	1.1	1460	1,606	2.09

* (Subcommittee on Feed Intake, Committee on Animal Nutrition, National Research Council 1987)

+ Average weight at slaughter

++ (Stalder, Engblom et al. 2009)

SAFETY ASSESSMENT OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED

Antibiotic Resistance

The final production strain does not contain any Antibiotic Resistance genes. The strain is susceptible to antibiotics and to anti-fungals. When tested, the genetic changes introduced into the Inactivated Modified *Saccharomyces cerevisiae* do not affect antifungal susceptibility.

A literature search did not produce any articles related to resistance to antibiotics. Since the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, data were provided for the species in general based on the rationale that the gene modification to the organism was not shown through a literature search to produce an effect or yield different results from the parental strain. For this reason, Expert Panel concurs with DSM that it is appropriate to use the parental strain, *Saccharomyces cerevisiae,* as a surrogate strain for gathering information and assessing the effect of the modified strain on antibiotic resistance and tolerance to metals and pesticides.

Based on the absence of demonstrated adverse effects for the parental strain and for the inserted intergeneric sequence, it is reasonable to conclude that the modified strain is not expected to be any different from other well-known *Saccharomyces cerevisiae* strains commonly found in nature.

Action as a Potential Vector for Pathogens

A search of the literature did not identify any articles demonstrating (b)(4) permits the production strain to act as a vector of pathogens. A null result was not unexpected since (b)(4) has been used in industry and food processing for years without any adverse effect. There are no studies that the submitter could locate that would indicate the donor strain itself, (b)(4) acts as a vector for pathogens. Because the gene modification to the organism was not shown through a literature search to yield different results from the parental strain, surrogate information on the recipient strain is offered for the purpose of evaluating the anticipated behavior of the production strain. Based on the information provided in EPA's February 1997 Final Risk Assessment (the Environmental Protection Agency 1997), the production strain is not expected to be a vector for pathogens. The production strain is not expected to act as a vector of any pathogen such as *Escherichia coli* or *Clostridium botulinum*.

Safety Assessment of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has an extensive history of use in food processing. Also known as Baker's Yeast or Brewer's Yeast, this organism has been used from as early as the Stone Age (about 9000 BC) as leavening for bread and as a fermenter of alcoholic beverages (Tucker, Woods 1995). But it was only between 1857 and 1863 that Louis Pasteur demonstrated the role played by yeasts, as the micro-organism responsible for fermentation. He noted at that time that one type of living cells was responsible for the fermentation of bread, beer, wine, cider corresponding to a population of a microscopic fungus, *Saccharomyces cerevisiae*. He discovered that a number of varieties of *Saccharomyces cerevisiae* exist in nature and are more or less adapted to the different fermentations (Pretorius 2000). *Saccharomyces cerevisiae* is considered Generally Recognized as Safe through its use in the brewing, baking and

winemaking industry and is the subject of several GRAS Notices, 120, 175, 239, 260, 284, 350, 353 (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175, CFSAN / Office of Food Additive Safety 2008, GRN 000260, CFSAN / Office of Food Additive Safety 2008, GRN 000239, CFSAN / Office of Food Additive Safety 2009, GRN 000284, CFSAN / Office of Food Additive Safety 2010, GRN 000350, CFSAN / Office of Food Additive Safety 2010, GRN 000353).

In food production today the use of yeasts is not limited to the production of bread or fermented beverages. Since the 1930s yeast extract products were developed to enhance the flavor of a variety of products such as soups, sauces and ready-to-eat meals. Moreover, because of their nutritional characteristics (protein, vitamin, mineral and amino acid content), preparations of living cells or yeast cell walls have been commercialized as food supplements. Yeast is recommended for dietary supplementation for patients with diabetes type 2, diarrhea, high cholesterol and fatigue due to its high chromium and vitamin B content (see www.vitacost.com). In addition, the organism is widely used for the production of macromolecular cellular components such as lipids, proteins, enzymes, and vitamins (see www.vitacost.com; Moyad 2007, Moyad 2008).

Saccharomyces cerevisiae is a ubiquitous yeast. It is found on our bodies and in the air that we breathe, and it is naturally present on and in foods that man and animals eat regularly.

In its safety evaluation of carbohydrase from *Saccharomyces cerevisiae*, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered that *Saccharomyces cerevisiae* belongs to the group of micro-organisms traditionally accepted as constituents of foods or normally used in the preparation of foods (Joint FAO/WHO Expert Committee on Food Additives 1971).

In 1997, the US Environmental Protection Agency (EPA) issued their safety assessment of *Saccharomyces cerevisiae* and concluded that "*S. cerevisiae* is an organism which has an extensive history of safe use. Despite considerable use of the organism in research and the presence of *S. cerevisiae* in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the human had a debilitating condition. Tests for the factors associated with the virulence of yeasts (i.e., phospholipases) indicate that this organism is nonpathogenic. The organism has not been shown to produce toxins to humans." (Environmental Protection Agency, 1997) *Saccharomyces cerevisiae* was therefore included as a recipient microorganism at § 725.420 for the tiered exemption.

According to scientific experts, a nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism is defined as "one that is very unlikely to produce disease under ordinary circumstances." (Pariza, Foster 1983).

The European Food Safety Agency (EFSA) concluded that baker's yeast (*Saccharomyces cerevisiae*) fits this definition of nontoxigenicity and nonpathogenicity and that it is also not a major food allergen. EFSA therefore gave baker's yeast a Qualified Presumption of Safety (QPS) status (Opinion of the Scientific Committee, 2007). This means that in Europe, baker's yeast can be safely used in food and feed production and is exempt from the need for further safety assessment.

The FDA considers *Saccharomyces cerevisiae* and several derived products safe for consumption. Indeed, FDA has approved dried yeast as an ingredient for food (21 C.F.R. §172.896), and baker's yeast extract has been affirmed by the FDA as a GRAS flavoring agent and adjuvant (21 C.F.R. §184.1983). The FDA has also approved various yeast-derived products for their use in food. These include Baker's yeast protein (21 C.F.R. §172.325), Yeast-malt sprout extract (21 C.F.R. §172.590) and Baker's yeast glycan (21 C.F.R. §172.898).

In addition, GRAS notifications have been submitted and accepted by FDA with no questions for the use of genetically modified yeasts as starter cultures for wine (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175).

Safety Considerations Due to the Nature of Modifications of *Saccharomyces cerevisiae*

The US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420). This exemption is permitted because genetically engineered strains of the species were found to have no adverse effects on man or the environment. They also determined that the introduction of intergeneric material will not increase the potential for adverse effects, provided that the genetic material is limited in size, well characterized, free of certain sequences and poorly mobilizable (Environmental Protection Agency, 1997).

For DSM's Modified *Saccharomyces cerevisiae*:

- the only additional heterologous genetic material was (b)(4) and the coding sequence of the inserted gene is limited in size;
- the additional genetic material is completely characterized as it was made synthetically;
- the additional genetic material by default is free from sequences of concern, and the additional genetic material is poorly metabolizable as it is integrated in the genome of the host and lacks sequences which would allow it to be mobilized;
- no antibiotic resistance genes are present;
- no toxigenic genes were inserted;
- no antibiotic production genes were inserted.

The gene insertion was not random but targeted and the locations are known as illustrated in sections 2.3.5 and 2.4 of this dossier. The entire genome of *Saccharomyces cerevisiae* is known and it is free of toxin producing DNA sequences (see http://www.yeastgenome.org/ for the entire genome). It is not possible to induce the formation of a new attribute utilizing the techniques described in the organism construction sections of the dossier.

The Modified *Saccharomyces cerevisiae* that is the subject of this GRAS Notice is also the subject of the Microbial Commercial Activity Notice J13-0007 which was dropped from their review in June 2013. DSM also assessed the safety of the Inactivated Modified Yeast as it relates to its use in foods according to the decision tree guidelines developed by Pariza and Johnson (2001) for food enzymes and later modified by Pariza and Cook (2010) for animal feed

enzymes derived from GE organisms. The test article was shown to be acceptable for feed use. In addition, the entire organism is boiled in acidic aqueous fermentation broth during the distillation step in biofuel production and destroyed.

The Expert Panel critically evaluated published data and a third party assessment by the European Food Safety Agency (Opinion of the Scientific Committee, 2007 on the safety of *Saccharomyces cerevisiae*). Particular attention in the EFSA focused on reports of the role of *S. cerevisiae* as an aetiological agent of invasive infection in "fragile" populations. EFSA concluded "*The body of knowledge is considered as sufficient (long history of safe use) with only 92 cases of pathogenic cases involving S. cerevisiae reported in total (15 cases diagnosed before 1990); all patients had at least one condition facilitating the opportunistic development of S. cerevisiae. S. bayanus and S. pastorianus are used in wine and beer production. There are no foodborne infection issues for these species.* On the basis of their assessment, EFSA classified *Saccharomyces cerevisiae* as a QPS (Qualified Presumption as Safe) organism, a non-pathogen and not a major food allergen. *Saccharomyces* has been an approved component of feed since at least 1951 and there are no literature reports that identify *Saccharomyces cerevisiae* as toxic for animals. As stated earlier, this means that it can be safely used in food and feed without additional safety testing.

DSM also performed a comprehensive literature search and confirmed that although the cases of human infection with *Saccharomyces cerevisiae* are rare, some isolates have pathogenic potential. Some clinical isolates are notably known to be virulent in humans. In addition, several reports evaluating the safety of commercial *Saccharomyces cerevisiae* strains have clearly identified the subtype *boulardii* as a potential pathogen, in particular towards immunocompromised patients (Graf and Gavazzi, 2007).

Although very few scientific reports are available on potential adverse effects of *Saccharomyces cerevisiae* for animals, the few toxicity studies that were performed on rats, mice or monkeys with the yeast or with the yeast biomass do not suggest that these animals display a stronger sensitivity towards *Saccharomyces cerevisiae* than humans.

Recent efforts have focused on attempting to characterize the virulence traits related to *Saccharomyces cerevisiae, thus* enabling the means to better identify the potential pathogenic strains. The phenotypic traits of virulence of pathogenic strains are believed to be due to their ability to grow at high temperature (38-42◦C), their adherence and their ability to invade host cells, and their ability to produce and secrete degradative enzymes (i.e. proteinase and phospholipase). This search did not identify any reports of isolates of *Saccharomyces cerevisiae* able to produce toxins against animals or humans, although several studies have shown the ability of the yeast to produce toxins against other yeasts (the so-called killer toxins).

The Expert Panel concluded that DSM's literature search does not alter the conclusions previously made by several bodies of experts that *Saccharomyces cerevisiae* can be considered as a safe microorganism and a non-pathogen, although some of the yeast isolates should be regarded as opportunistic pathogens of low virulence.

Presence of Methyl Glyoxal

In metabolically active yeast cells, methylglyoxal may be formed during normal cell metabolism. Methylglyoxal synthase catalyzes the reaction from dihydroxyacetonephosphate to methylglyoxal. Hashimoto et. al (1997) found that genetically engineered *Saccharmoycese*

cerevisiae strain DKD-5D-H produced an increased amount of methylglyoxal, a toxic 2-oxoaldehyde, compared to the wild-type yeast cells. This increase was reportedly due to the genetic manipulations involving the enzymes phosphoglucose isomerase, phosphofructokinase and triosephosphate isomerase.

In the Inactivated Modified Yeast, overexpression of the *GLO1*-gene is brought about. To this end, the *Saccharomyces cerevisiae GLO1*-gene is expressed under control of the PGK1-promoter. The *GLO1*-gene encodes monomeric glyoxalase I, which catalyzes the detoxification of methylglyoxal (a by-product of glycolysis) via condensation with glutathione to produce S-D-lactoylglutathione. The Expert Panel noted that DSM explains in the GRAS dossier that overexpression of the GLO1-gene helps in lowering the intracellular glyoxal levels and therefore, this is not a concern.

Human Exposure

Human exposure is anticipated to be limited to individuals handling the material during packaging at the yeast production facility, the bio-ethanol facility, in the manufacture of feed, on the farm, and from consumption of the target animals. The use of personal protective equipment and compliance with normal occupational safety practices will minimize exposure.

No toxicokinetic studies on *Saccharomyces cerevisiae* were identified by DSM. However, *Saccharomyces cerevisiae* will be metabolized during animal digestion into essential compounds consisting mainly of proteins and carbohydrates. Amino acids and peptides are important building blocks in the human body. Carbohydrates are indispensable for storage or transport of energy, and they also play a role in immune function, blood clotting and development.

The Expert Panel concurred and concluded that *Saccharomyces cerevisiae* is in general considered to be of very low toxicity for humans. This conclusion is based on human evidence coming from a long history of safe use, on its composition, and on the fact that it is readily biodegradable in the human gastro-intestinal tract. In the public domain, toxicity studies on *Saccharomyces cerevisiae* are limited. However, due to low risk expected for man and the environment, the US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism is exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420).

Allergenicity Concerns

Inhalation allergy towards *Saccharomyces cerevisiae* has been described by Baldo and Baker (Baldo, Baker 1988). This type of allergy is predominantly observed in bakers.

Regarding oral allergy against *Saccharomyces cerevisiae,* only one possible case is known (Pajno, Passalacqua et al. 2005). An atopic 6-year old boy experienced generalized urticaria and asthma after eating pizza and bread fresh from the oven (within one hour. In its assessment of *Saccharomyces cerevisiae* for a classification as QPS (Qualified Presumption as Safe) organism, the European Food Safety Agency (EFSA) concluded that *Saccharomyces cerevisiae* is not a major food allergen (Opinion of the Scientific Committee, 2007).

In addition, DSM's also reported on their assessment of the impact of the modifications made on the *Saccharomyces cerevisiae* portion of DDGS was evaluated by applying the guidelines on testing the potential allergenicity of genetically modified foods as presented by the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (Food and Agriculture Organization of the United Nations/World Health Organization 2001, Food and Agriculture Organization of the United Nations/World Health Organization 2009).

The Inactivated Modified *Saccharomyces cerevisiae* has been engineered to (b)(4), but no foreign DNA sequences known to code for allergenic proteins were inserted into the organism.

Regarding the above, it is concluded that the inserted gene protein product has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

***Environmental* Impact**

The Expert Panel also noted and agreed that *Saccharomyces cerevisae* is exempted from TSCA Section 5 PreMarket Notification by the Environmental Protection Agency (40 CFR §725.420, the Environmental Protection Agency 1997). The EPA has reviewed and approved the use of this genetically engineered *Saccharomyces cerevisiae* for biofuel production under Microbial Commercial Activity Notice (MCAN) J11-0001. Another genetically engineered yeast, *Pichia pastoris*, has also been approved under MCAN J04-0003. DSM has filed a Tier 1 exemption notice with the EPA which was accepted by the agency in December 2011 and a Microbial Commercial Activity Notice which was accepted on 18 March 2013 and dropped from review on 12 June 2013.

Inactivated Modified *Saccharomyces cerevisiae* is a GRAS substance and per 21 CFR §25.32, foods, food additives and color additives, including GRAS substances, are categorically excluded from the requirement to provide an environmental impact statement or an environmental assessment.

Inactivated Modified *Saccharomyces cerevisiae* will be added directly to animal food and is intended to remain in the food through ingestion by animals and it is not intended to replace macronutrients in animal food.

There is no information on the ecotoxicity of Modified *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* in the public domain. However, because of the macro-composition of Modified *Saccharomyces cerevisiae*, consisting mainly of proteins and carbohydrates, it can be expected that the substance is readily biodegradable. Therefore, no toxicity for invertebrates, aquatic plants and fish is expected, since the substance will be degraded by bacteria in the water. In addition, as was previously indicated, *Saccharomyces cerevisiae* has been widely fed to poultry, cattle and other livestock due to its nutritious properties and is thus of very low toxicity. It is also used as nutritious mixture in *in vivo* ecotoxicity tests, meaning that many animals in ecotoxicity studies are fed or supplemented with *Saccharomyces cerevisiae*.

CONCLUSION

We, the undersigned independent qualified members of the Expert Panel, have individually and collectively critically evaluated the data and information summarized above, as well as other data and information that we deemed pertinent to the safety of the intended conditions of use of the genetically engineered pentose fermenting *Saccharomyces cerevisiae*, referred to in this document as Inactivated Inactivated Modified *Saccharomyces cerevisiae*, produced during bio-fuel production from plant- based materials and meeting appropriate established specifications is safe for consumption by production animals, minor species and pets. Modified *Saccharomyces cerevisiae* is manufactured following Good Large Scale Fermentation Practices and is in compliance with the applicable sections of 40 CFR §725. Inactivated Modified *Saccharomyces cerevisiae* will enter the animal food chain as a by-product of ethanol production from grains, such as corn, wheat, barley, rye and other forage materials. It is anticipated that the substance will be isolated from the stillage of the bio-fuel plant and sold as a nutritive ingredient or it will be a component of the dried stillage commonly referred to as 'dried distillers' grains' (DDG).

We further conclude that the proposed uses of Inactivated Modified *Saccharomyces cerevisiae* meeting appropriate feed-grade specifications presented in the supporting dossier and produced consistent with Good Large Scale Fermentation Practices, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

Mark E. Cook — Dec 12, 2013

Mark E. Cook, Ph.D.
University of Wisconsin (Panel Member)

Date

Stanley M. Tarka — 13 December 2013

Stanley M. Tarka, Ph.D.
The Tarka Group, Inc. (Chair)

Date

John A. Thomas — Dec. 11, 2013

John A. Thomas, Ph.D., F.A.T.S.
Indiana University School of Medicine (Panel Member)

Date

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Section 172: FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION

§ 172.325 - Bakers yeast protein.

§ 172.590 - Yeast-malt sprout extract.

§ 172.896 - Dried yeasts.

§ 172.898 - Bakers yeast glycan.

184: DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

§ 184.1983 - Bakers yeast extract.

(b)(4)

SUBMISSION
CONTINUED

IN

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in accordance with copyright laws

Annex 7
Lab procedures -inactivation

RAW DATA

Distillation column

working volume	50% full

volume was calculated based on the dimentions from P&ID (pipeline & instrumentation diagram)

working volume	245.07	gal

	#	Temp. °F	Temp. °C	Flowrate (ft3/hr)	Flowrate (g/min)
Feed	25	189	87.2	204.7	25.5
Out	26	228	108.9	196.4	24.5
Average			98.1	200.6	25.0
Residence time	9.8	min			

Experimental plan for yeast killing — Trial #

Trial#	1	2	3	4	5	6	7 (control)
Temp. °C	88	90	95	80	90	95	n/a
Time (min)	5	5	5	9	9	9	n/a

At each condition: do triplate for counting

total plate	21

Yeast loads

g/L	OD600
1.3	2.6

Make yeast suspension

OD600	Dilution	Actual OD600
21.0	10	29.045

	ml	mL	mL
Target OD600	Total volume	Suspension Volume	R/O water
2.6	100	9.0	91.0

OD600	CFU	0.1 mL	Dilution	Expected CFU
1	2.00E+07	2.00E+06	10000	2.00E+02

Dilution for counting — Trial #

	1	2	3	4	5	6	7	no inoculation / negativ control
Dilution factor	10	10	10	10	10	10	10000	
	10	10	10	10	10	10	10000	
	1	1	1	1	1	1	10000	
	1	1	1	1	1	1	n/a	
	1	1	1	1	1	1	n/a	

	1	2	3	4	5	6	7	no inoculation / negativ control
Labeling	1.A	2.A	3.A	4.A	5.A	6.A	7.A	
	1.B	2.B	3.B	4.B	5.B	6.B	7.B	
	1.C	2.C	3.C	4.C	5.C	6.C	7.C	
	1.D	2.D	3.D	4.D	5.D	6.D	n/a	
	1.E	2.E	3.E	4.E	5.E	6.E	n/a	

	1	2	3	4	5	6	7	no inoculation / negativ control
Counted colony #	0	0	0	0	0	0	218	0
	0	0	0	0	0	0	222	
	0	0	0	0	0	0	209	
	0	0	0	0	0	0	n/a	
	0	0	0	0	0	0	n/a	
Average	0	0	0	0	0	0	216	

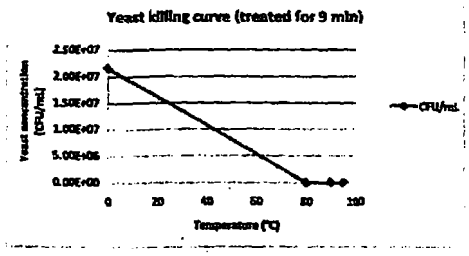
Initial yeast CFU/mL						
2.2E+07						
	1 (80°C/5min)	2 (90°C/5min)	3 (95°/5min)	4 (80°C/9min)	5 (90°C/9min)	6 (95°C/9min)
CFU/mL	0	0	0	0	0	0

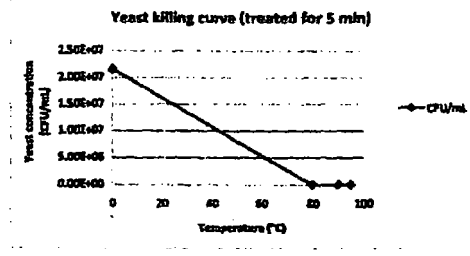
Treated for 5 min

Temperature (°C)	CFU/mL
0	2.16E+07
80	0
90	0
95	0

Treated for 9 min

Temperature (°C)	CFU/mL
0	2.16E+07
80	0
90	0
95	0





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Annex 12
DDG Amino Acid Analysis Statistics

Statistics – DDG Amino Acid Profile

Amino Acid Concentration % Dry Basis

Amino Acid Concentrations (%) Dry Basis in Samples performed by University of IL								
ID	503	507	598	599	923	908	928	924
Amino Acid	*Modified Saccharo myces cervisiae*	*Modified Saccharo myces cervisiae*	*Modified Saccharo myces cervisiae*	*Modified* Saccharo myces cervisiae	Conventional Saccharomyces cervisiae	Conventional Saccharomyces cervisiae	Conventional Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
ASP	2.85	2.67	3.01	2.68	2.04	1.96	2.06	1.83
THR	1.67	1.59	1.75	1.58	1.19	1.17	1.18	1.08
SER	2.07	1.96	2.26	2.05	1.43	1.30	1.31	1.29
GLU	7.13	6.79	8.03	7.33	4.96	3.79	4.11	3.93
PRO	3.57	3.33	3.78	3.40	2.45	1.99	2.09	1.95
GLY	1.72	1.55	1.73	1.54	1.24	1.28	1.35	1.20
ALA	3.28	3.10	3.46	3.12	2.27	2.03	2.13	1.99
CYS	0.83	0.79	0.88	0.81	0.61	0.53	0.52	0.52
VAL	2.31	2.15	2.37	2.11	1.58	1.66	1.74	1.45
MET	0.96	0.90	1.03	0.92	0.67	0.57	0.60	0.54
ILE	1.80	1.71	1.87	1.68	1.22	1.19	1.25	1.10
LEU	5.65	5.50	6.05	5.44	3.83	3.36	3.51	3.15
TYR	1.84	1.74	1.95	1.73	1.16	1.21	1.22	1.04
PHE	2.36	2.26	2.49	2.22	1.62	1.68	1.71	1.40
LYS	1.20	1.03	1.25	1.03	0.97	0.84	0.88	0.81

Amino Acid Concentration of Modified *Saccharomyces cervisiae* samples normalized for sample protein level

Amino Acid Concentrations (%) Dry Basis in Samples performed by University of IL								
ID	503	507	598	599	923	908	928	924
Amino Acid	*Modified Saccharo myces cervisiae*	*Modified Saccharo myces cervisiae*	*Modified Saccharo myces cervisiae*	*Modified* Saccharo myces cervisiae	Conventional Saccharomyces cervisiae	Conventional Saccharomyces cervisiae	Conventional Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
ASP	1.91	1.79	2.02	1.80	2.04	1.96	2.06	1.83
THR	1.12	1.06	1.17	1.06	1.19	1.17	1.18	1.08
SER	1.39	1.31	1.51	1.37	1.43	1.30	1.31	1.29
GLU	4.78	4.55	5.38	4.91	4.96	3.79	4.11	3.93
PRO	2.39	2.23	2.53	2.28	2.45	1.99	2.09	1.95
GLY	1.15	1.04	1.16	1.03	1.24	1.28	1.35	1.20
ALA	2.20	2.08	2.32	2.09	2.27	2.03	2.13	1.99
CYS	0.56	0.53	0.59	0.55	0.61	0.53	0.52	0.52
VAL	1.55	1.44	1.59	1.42	1.58	1.66	1.74	1.45
MET	0.64	0.60	0.69	0.62	0.67	0.57	0.60	0.54
ILE	1.21	1.14	1.25	1.13	1.22	1.19	1.25	1.10
LEU	3.78	3.69	4.05	3.65	3.83	3.36	3.51	3.15
TYR	1.23	1.17	1.31	1.16	1.16	1.21	1.22	1.04
PHE	1.58	1.51	1.67	1.49	1.62	1.68	1.71	1.40
LYS	0.80	0.69	0.83	0.69	0.97	0.84	0.88	0.81

The adjusted concentrations were used in the following statistical analysis.

Statistical analysis performed with Microsoft Excel built in data analysis tools.

ASP

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.906979	2.04
1.789081	1.96
2.015038	2.06
1.798943	1.83

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	7.510041	1.87751	0.011258
Conventional Saccharomyces cervisiae	4	7.89	1.9725	0.010892

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.018046	1	0.018046	1.629453	0.248958	5.987378
Within Groups	0.06645	6	0.011075			
Total	0.084496	7				

THR

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.118	1.19
1.065	1.17
1.173	1.18
1.055	1.08

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	4.411	1.10275	0.002958
Conventional Saccharomyces cervisiae	4	4.62	1.155	0.002567

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00546	1	0.00546	1.976784	0.209345	5.987378
Within Groups	0.016573	6	0.002762			
Total	0.022033	7				

SER

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.388	1.43
1.311	1.3
1.511	1.31
1.374	1.29

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	5.584	1.396	0.006999
Conventional Saccharomyces cervisiae	4	5.33	1.3325	0.004292

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.008065	1	0.008065	1.428483	0.277105	5.987378
Within Groups	0.033873	6	0.005645			
Total	0.041938	7				

GLU

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
4.778	4.96
4.549	3.79
5.38	4.11
4.91	3.93

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	19.617	4.90425	0.122838
Conventional Saccharomyces cervisiae	4	16.79	4.1975	0.275558

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.998991	1	0.998991	5.015067	0.066399	5.987378
Within Groups	1.195188	6	0.199198			
Total	2.194179	7				

PRO

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
2.389	2.45
2.23	1.99
2.53	2.09
2.28	1.95

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	9.429	2.35725	0.01767
Conventional Saccharomyces cervisiae	4	8.48	2.12	0.051867

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.112575	1	0.112575	3.237852	0.122053	5.987378
Within Groups	0.208611	6	0.034768			
Total	0.321186	7				

GLY

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.154	1.24
1.035	1.28
1.159	1.35
1.034	1.2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	4.382	1.0955	0.004966
Conventional Saccharomyces cervisiae	4	5.07	1.2675	0.004092

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.059168	1	0.059168	13.06521	0.011169	5.987378
Within Groups	0.027172	6	0.004529			
Total	0.08634	7				

ALA

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
2.2	2.27
2.079	2.03
2.317	2.13
2.09	1.99

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	8.686	2.1715	0.012394
Conventional Saccharomyces cervisiae	4	8.42	2.105	0.015567

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.008845	1	0.008845	0.632646	0.45669	5.987378
Within Groups	0.083881	6	0.01398			
Total	0.092726	7				

CYS

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
0.555	0.61
0.529	0.53
0.59	0.52
0.545	0.52

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	2.219	0.55475	0.000667
Conventional Saccharomyces cervisiae	4	2.18	0.545	0.0019

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00019	1	0.00019	0.148135	0.713597	5.987378
Within Groups	0.007701	6	0.001283			
Total	0.007891	7				

VAL

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.549	1.58
1.44	1.66
1.59	1.74
1.416	1.45

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	5.995	1.49875	0.00705
Conventional Saccharomyces cervisiae	4	6.43	1.6075	0.015292

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.023653	1	0.023653	2.117377	0.195877	5.987378
Within Groups	0.067026	6	0.011171			
Total	0.090679	7				

MET

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
0.6	0.67
0.6	0.57
0.69	0.6
0.62	0.54

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	2.51	0.6275	0.001825
Conventional Saccharomyces cervisiae	4	2.38	0.595	0.0031

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002113	1	0.002113	0.857868	0.390071	5.987378
Within Groups	0.014775	6	0.002463			
Total	0.016888	7				

ILE

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.205	1.22
1.144	1.19
1.252	1.25
1.126	1.1

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	4.727	1.18175	0.003336
Conventional Saccharomyces cervisiae	4	4.76	1.19	0.0042

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000136	1	0.000136	0.036125	0.855524	5.987378
Within Groups	0.022609	6	0.003768			
Total	0.022745	7				

LEU

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
3.784	3.83
3.687	3.36
4.052	3.51
3.647	3.15

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	15.17	3.7925	0.033238
Conventional Saccharomyces cervisiae	4	13.85	3.4625	0.081825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.2178	1	0.2178	3.785763	0.099651	5.987378
Within Groups	0.345188	6	0.057531			
Total	0.562988	7				

TYR

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.235	1.16
1.167	1.21
1.31	1.22
1.161	1.01

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	4.873	1.21825	0.004868
Conventional Saccharomyces cervisiae	4	4.6	1.15	0.0094

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009316	1	0.009316	1.305915	0.296676	5.987378
Within Groups	0.042803	6	0.007134			
Total	0.052119	7				

PHE

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.58	1.62
1.51	1.68
1.67	1.71
1.49	1.4

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	6.25	1.5625	0.006625
Conventional Saccharomyces cervisiae	4	6.41	1.6025	0.019625

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0032	1	0.0032	0.24381	0.639021	5.987378
Within Groups	0.07875	6	0.013125			
Total	0.08195	7				

LYS

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
0.8	0.97
0.69	0.84
0.835	0.88
0.687	0.81

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	3.012	0.753	0.005753
Conventional Saccharomyces cervisiae	4	3.5	0.875	0.004833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.029768	1	0.029768	5.624032	0.055407	5.987378
Within Groups	0.031758	6	0.005293			
Total	0.061526	7				

HIS

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
0.738	0.88
0.674	0.79
0.784	0.81
0.665	0.79

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	2.861	0.71525	0.003157
Conventional Saccharomyces cervisiae	4	3.27	0.8175	0.001825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.02091	1	0.02091	8.39441	0.027431	5.987378
Within Groups	0.014946	6	0.002491			
Total	0.035856	7				

ARG

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
1.45	1.38
1.11	1.53
1.27	1.58
1.07	1.37

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	4.9	1.225	0.029967
Conventional Saccharomyces cervisiae	4	5.86	1.465	0.011233

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1152	1	0.1152	5.592233	0.055917	5.987378
Within Groups	0.1236	6	0.0206			
Total	0.2388	7				

TRP

Modified Saccharomyces cervisiae	Conventional Saccharomyces cervisiae
0.23	0.26
0.18	0.26
0.23	0.27
0.178	0.23

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	0.818	0.2045	0.000868
Conventional Saccharomyces cervisiae	4	1.02	0.255	0.0003

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.005101	1	0.005101	8.736226	0.025424	5.987378
Within Groups	0.003503	6	0.000584			
Total	0.008604	7				

Annex 13
DDG AA Digestability Statistics

Statistical Analysis of DDG Digestibility Data

True Amino Acid Digestibility (%) performed by University of IL

	503	*507*	*598*	*599*	*923*	*908*	*928*	*924*
Amino Acid	Modified *Saccharomyces cervisiae*	Modified *Saccharomyces cervisiae*	Modified *Saccharomyces cervisiae*	Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
ASP	73.6	75.7	71.02	71.15	74.42	68.35	68.26	65.66
THR	75.58	75.65	70.37	71.44	74.5	71.41	69.83	68.88
SER	80.07	82.34	77.83	76.64	82.92	75.68	72.47	71.05
GLU	84.08	85.39	83.88	83.27	86.58	77.99	79.23	79.18
PRO	82.18	81.45	80.16	79.37	85.02	77.9	77.12	78.66
ALA	84.81	85.45	83.11	83.26	85.45	79.85	79.38	80.89
CYS	68.35	71.03	67.24	67.84	74.89	67.04	64.85	64.14
VAL	80.43	80.33	76.29	76.67	80.53	76.18	75.99	76.2
MET	86.17	86.99	85.07	84.31	86.55	81.1	80.42	80.71
ILE	81.51	81.77	77.01	78.02	80.46	78.14	76.96	76.22
LEU	87.76	88.07	86.02	86.1	88.94	86.48	85.46	86
TYR	87.87	88.5	85.13	85.24	85.91	82.08	80.11	81.77
PHE	85.75	86.1	82.96	83.58	85.03	82.47	81.14	82.32
LYS	63.04	60.33	52.31	52.74	57.94	53.14	48.04	42.62
HIS	77.19	77.3	74.89	73.93	79.95	73.36	72.81	76.03
ARG	86.59	86.48	81.23	82.85	85.77	81.94	80.76	80.74
TRP	88.53	89.37	88.64	85.35	91.27	84.23	87.73	88.53

ASP

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
73.60	74.42
75.70	68.35
71.02	68.26
71.15	65.66

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	291.47	72.8675	4.974225
Conventional Saccharomyces cervisiae	4	276.69	69.1725	13.79436

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27.30605	1	27.30605	2.909761	0.138922	5.987378
Within Groups	56.30575	6	9.384292			
Total	83.6118	7				

THR

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
75.58	74.50
75.65	71.41
70.37	69.83
71.44	68.88

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	293.04	73.26	7.586333
Conventional Saccharomyces cervisiae	4	284.62	71.155	6.061767

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.86205	1	8.86205	1.29865	0.297902	5.987378
Within Groups	40.9443	6	6.82405			
Total	49.80635	7				

SER

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
80.07	82.92
82.34	75.68
77.83	72.47
76.64	71.05

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	316.88	79.22	6.348467
Conventional Saccharomyces cervisiae	4	302.12	75.53	28.02287

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27.2322	1	27.2322	1.584588	0.254861	5.987378
Within Groups	103.114	6	17.18567			
Total	130.3462	7				

GLU

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
84.08	86.58
85.39	77.99
83.88	79.23
83.24	79.18

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	336.59	84.1475	0.814492
Conventional Saccharomyces cervisiae	4	322.98	80.745	15.46057

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	23.15401	1	23.15401	2.845337	0.142612	5.987378
Within Groups	48.82518	6	8.137529			
Total	71.97919	7				

PRO

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
82.18	85.02
81.45	77.9
80.16	77.12
79.37	78.66

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	323.16	80.79	1.593667
Conventional Saccharomyces cervisiae	4	318.7	79.675	13.09263

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.48645	1	2.48645	0.338608	0.581822	5.987378
Within Groups	44.0589	6	7.34315			
Total	46.54535	7				

ALA

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
84.81	85.45
85.45	79.85
83.11	79.38
83.26	80.89

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	336.63	84.1575	1.333025
Conventional Saccharomyces cervisiae	4	325.57	81.3925	7.715092

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.29045	1	15.29045	3.379808	0.11563	5.987378
Within Groups	27.14435	6	4.524058			
Total	42.4348	7				

CYS

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
68.35	74.89
71.03	67.04
67.24	64.85
67.84	64.14

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	274.46	68.615	2.7979
Conventional Saccharomyces cervisiae	4	270.92	67.73	24.30807

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.56645	1	1.56645	0.11558	0.745465	5.987378
Within Groups	81.3179	6	13.55298			
Total	82.88435	7				

VAL

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
80.43	80.53
80.33	76.18
76.29	75.99
76.67	76.2

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	313.72	78.43	5.095733
Conventional Saccharomyces cervisiae	4	308.9	77.225	4.863633

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.90405	1	2.90405	0.58318	0.474017	5.987378
Within Groups	29.8781	6	4.979683			
Total	32.78215	7				

MET

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
86.17	86.55
86.99	81.1
85.07	80.42
84.31	80.71

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	342.54	85.635	1.399033
Conventional Saccharomyces cervisiae	4	328.78	82.195	8.506967

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	23.6672	1	23.6672	4.778357	0.071473	5.987378
Within Groups	29.718	6	4.953			
Total	53.3852	7				

ILE

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
81.51	80.46
84.77	78.14
77.01	76.96
78.02	76.22

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	321.31	80.3275	12.48816
Conventional Saccharomyces cervisiae	4	311.78	77.945	3.436367

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.35261	1	11.35261	1.425802	0.277512	5.987378
Within Groups	47.77358	6	7.962262			
Total	59.12619	7				

LEU

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
87.76	88.94
88.07	86.48
86.02	85.46
86.1	86

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	347.95	86.9875	1.164092
Conventional Saccharomyces cervisiae	4	346.88	86.72	2.364

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.143113	1	0.143113	0.081127	0.785351	5.987378
Within Groups	10.58428	6	1.764046			
Total	10.72739	7				

TYR

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
87.87	85.91
88.5	82.08
85.13	80.11
85.24	81.77

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	346.74	86.685	3.068167
Conventional Saccharomyces cervisiae	4	329.87	82.4675	6.015092

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	35.57461	1	35.57461	7.833007	0.031215	5.987378
Within Groups	27.24978	6	4.541629			
Total	62.82439	7				

PHE

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
85.75	85.03
86.1	82.47
82.96	81.14
83.58	82.32

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	338.39	84.5975	2.434158
Conventional Saccharomyces cervisiae	4	330.96	82.74	2.684467

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.900612	1	6.900612	2.696276	0.151691	5.987378
Within Groups	15.35588	6	2.559313			
Total	22.25649	7				

LYS

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
63.04	57.94
60.33	53.14
52.31	48.04
52.74	42.62

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	228.42	57.105	29.22337
Conventional Saccharomyces cervisiae	4	201.74	50.435	43.4841

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	88.9778	1	88.9778	2.447556	0.168743	5.987378
Within Groups	218.1224	6	36.35373			
Total	307.1002	7				

HIS

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
77.19	79.95
77.3	73.36
74.89	72.81
73.93	76.03

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	303.31	75.8275	2.834692
Conventional Saccharomyces cervisiae	4	302.15	75.5375	10.63116

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1682	1	0.1682	0.024982	0.879598	5.987378
Within Groups	40.39755	6	6.732925			
Total	40.56575	7				

ARG

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
86.59	85.77
86.48	81.94
81.23	80.76
82.85	80.74

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	337.15	84.2875	7.174425
Conventional Saccharomyces cervisiae	4	329.21	82.3025	5.658558

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.88045	1	7.88045	1.228156	0.310202	5.987378
Within Groups	38.49895	6	6.416492			
Total	46.3794	7				

TRP

Modified *Saccharomyces cervisiae*	Conventional *Saccharomyces cervisiae*
88.53	91.27
89.37	84.23
88.64	87.73
85.35	88.53

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Modified Saccharomyces cervisiae	4	351.89	87.9725	3.195625
Conventional Saccharomyces cervisiae	4	351.76	87.94	8.415067

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002113	1	0.002113	0.000364	0.985399	5.987378
Within Groups	34.83208	6	5.805346			
Total	34.83419	7				

Annex 14
Decision Tree Analysis

This analysis is based on the Decision Tree of MW Pariza and EA Johnson (2001): *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology, 33:173-186. The decision tree specifically designed for safety relative to animal feed is found in M.W. Pariza and M.E. Cook (2010); *Determining the safety of enzymes used in animal feed.* Regulatory Toxicology and Pharmacology, 56:332-342. *Italics=statements from the decision tree.*

1. *Is the production strain genetically modified?*

The production organism used is a genetically modified *Saccharomyces cerevisiae*. According to the decision tree, the production strain should be "nonpathogenic, non-toxigenic, and thoroughly characterized." *Saccharomyces cerevisiae* is a wild-type yeast that is common in food preparation and has a long history of safe use. (b)(4) A is a natural unmodified strain of *Saccharomyces cerevisiae* deposited in the (b)(4) archive as (b)(4). Wild type *Saccharomyces cerevisiae* is well known to be nonpathogenic and non-toxigenic. Various strains of *Saccharomyces cerevisiae* have been well characterized, and will there are functional differences among strains of *Saccharomyces cerevisiae* (b)(4), wild type *Saccharomyces cerevisiae* is generally recognized as a safe production species.

While the production organism is derived from a parent line that is nonpathogenic, non-toxigenic, and is well characterized, the production organism is genetically modified, hence, according to the decision tree, if yes, go to 2.

2. *Is the production strain modified using rDNA techniques?*

The parent strain was modified using recombinant DNA techniques as described in the GRAS document. According to the decision tree, if yes go to 3.

3. *Issues relating to the introduced DNA are addressed in 3a–3e.*

3a. *Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food or feed?*

The parent was modified to (b)(4). All the genes are native to *S. cerevisiae* with the exception of the (b)(4) from (b)(4). *S. cerevisiae* is a common feed ingredient and consequently yanimals have been exposed to the enzymes. (b)(4) is also known as (b)(4) which has been utilized for the production of (b)(4) since the 1970's. See (b)(4)

The yeast will be exposed to temperatures of approximately 80°C for several hours during ethanol recovery from the fermentation mash. In general, enzymes are inactivated at

temperatures >40°C. DSM provided experimental data showing that the organism is inactivated at distillation temperatures.

If yes, go to 3c. If no, go to 12. YES, assuming that the test article is *Saccharomyces cerevisiae*

3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?

3b does not apply.

3c. *Is the test article free of transferable antibiotic resistance gene DNA?*

Kuyper at al., 2004 used geneticin and hygromycin resistance in the formation of RWB217. One assume that the selection of spontaneous mutant RWB 218 continues to have geneticin and hygromycin resistance, although there was no evidence of their use in Kuyper at al., 2005. Statement made on page 11 of GRAS document is vague as to how this was accomplished, however greater detail was added for the removal of (b)(4) beginning section 2.3.5.

See Annex 4. Unclear if this was the production strain. According to wording page 54 section 2.11, it would appear all test were done using wild type? Also no test for resistant genes used.

Section 2.11. 1,2,3 provide evidence resistant genes are removed using PCR. It would appear that no antimicrobial and toxins would be present in the Modified *Saccharomyces cerevisiae* that were not found in the wildtype.

If yes, go to 3e. If no, go to 3d. YES

3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If no, go to 3e.

To our knowledge, (b)(4) is not used in human and animal medicine. (b)(4) and (b)(4) may still be approved for animal or human use. However since these genes have been removed, answer is no.

3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?

It would appear that the DNA differences between the wild type are restricted to enzymes of interest and it is well characterized (with comments above). Antimicrobial activities found in wild-type *Saccharomyces cerevisiae* are currently not used to treat animals and humans.

If yes, go to 4. If no, go to 12. YES

4. Is the introduced DNA randomly integrated into the chromosome?

Method of insertion was not random.

If yes, go to 5. If no, go to 6. NO

5. *Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?*

In this case the production strain is safe.

If yes, go to 6. If no, go to 7. YES

6. *Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?*

Saccharomyces cerevisiae used was from a safe lineage. Also since all the yeast are killed there would be no possibility for pathogenesis in the final product.

If yes, the test article is ACCEPTED. If no, go to 7. YES, The test article is accepted

7. Is the organism nonpathogenic?
If yes, go to 8. If no, go to 12.

8. Is the test article free of antibiotics?
If yes, go to 9. If no, go to 12.

9. Is the test article free of oral toxins known to be produced by other members of the same species?

If yes, go to 11. If no, go to 10.

10. Are the amounts of such toxins in the test article below levels of concern?

If yes, go to 11. If no, go to 12.

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety?
If yes, the test article is ACCEPTED.

12. An undesirable trait or substance may be present and the test article is not acceptable for feed use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.

Annex 15
Literature search

FOR INTERNAL USE ONLY

Results of a literature search on the safety of *Saccharomyces cerevisiae*

Literature was searched for scientific publications on any adverse effect, toxicological effect or safety issue of *Saccharomyces cerevisiae*. The databases Medline, BIOSIS and EMBASE as provided by the host STN were searched simultaneously. The genus name was used as such without adding any species indication. Usual terminology related to pathogenicity, toxicology or safety was used. The search strategy is shown here.

Search strategy

```
* * * * * * * * *   Welcome to STN International   * * * * * * * * * *

=> file medline, biosis, embase

=> s saccharomyces or baker?(w)yeast or brewer?(w)yeast
L1      307623 SACCHAROMYCES OR BAKER?(W) YEAST OR BREWER?(W) YEAST

=> s pathogen? or tox? or safe? or allergen?
L2     7681025 PATHOGEN? OR TOX? OR SAFE? OR ALLERGEN?

=> s l1 (L) l2
L3       13899 L1 (L) L2

=> s l1 (3a) l2
L4        2324 L1 (3A) L2

=> duplicate
ENTER REMOVE, IDENTIFY, ONLY, OR (?):remove
ENTER L# LIST OR (END):14
DUPLICATE PREFERENCE IS 'MEDLINE, BIOSIS, EMBASE'
KEEP DUPLICATES FROM MORE THAN ONE FILE?  Y/(N):n

L5         1821 DUPLICATE REMOVE L4 (503 DUPLICATES REMOVED)


=> s l4/ti

L6         408 L4/TI

=> duplicate
ENTER REMOVE, IDENTIFY, ONLY, OR (?):remove
ENTER L# LIST OR (END):16
DUPLICATE PREFERENCE IS 'MEDLINE, BIOSIS, EMBASE'
KEEP DUPLICATES FROM MORE THAN ONE FILE?  Y/(N):n
PROCESSING COMPLETED FOR L6
L7          184 DUPLICATE REMOVE L6 (224 DUPLICATES REMOVED)


=> d bib,ab 1-184
```

The abstracts of all 184 papers are shown in the appendix. The most relevant publications are categorized as shown here. The papers that were not selected primarily deal with adverse effects of substances against Saccharomyces, an obvious difficulty of searching.

Direct adverse effects of *S. cerevisisae*

Toxicology towards animals (and one plant)

1. In vivo virulence of commercial Saccharomyces cerevisiae strains with pathogenicity -associated phenotypical traits.

R. de Llanos, S. Llopis, G. Molero, A. Querol, C. Gil and M. T. Fernandez-Espinar.

Departamento de Biotecnologia, Instituto de Agroquimica y Tecnologia de los Alimentos CSIC, PO Box 73, 46100 Burjassot, Valencia, Spain.

Int. J. Food Microbiol. 2011, 144 (3): 393-399

English

ABSTRACT Two commercial Saccharomyces cerevisiae strains, a baker's strain and the bio-therapeutic agent Ultralevure, have been proposed as a possible exogenous source of human colonization (de Llanos et al., 2004, 2006a). Moreover, these strains express phenotypical traits associated to pathogenicity (de Llanos et al., 2006b). Taking into account that both commercial preparations represent an important source of living S. cerevisiae cells we have performed an in vivo study to evaluate whether there is a potential safety risk to humans. Their virulence was compared with that of other commercial strains with less virulent traits, and with clinical isolates, using two murine models (BALB/c and DBA/2N mice). Burden determination in the brain and kidneys showed that the ability to disseminate, colonize and persist was manifested not only by clinical isolates but also by commercial strains. Among these, the baker's strain and Ultralevure were able to cause the death of BALB/c mice at rates similar to those shown by two of the clinical isolates. These results highlight the pathogenic potential of these strains and show that four-week-old BALB/c mice are an appropriate murine model to study the virulence of yeasts with low or moderate pathogenicity. Furthermore, we have shown the positive effect of an immunosuppressive therapy with cyclophosphamide in the virulence of the baker's strains and Ultralevure but not in the rest of the commercial strains under study. The data suggest that although S. cerevisiae has always been considered a GRAS microorganism, commercial preparations should include only those strains shown to be safe in order to minimize complications in risk groups.

*****

2. Nutritional and toxicological evaluation of Saccharomyces cerevisae fermented cassava flour.

G. Oboh and A. A. Akindahunsi.

Fed Univ Technol, Dept Biochem, PMB 704, Akure, Nigeria goboh2001@yahoo.com

Journal of Food Composition and Analysis 2005, 18 (7): 731-738

English

ABSTRACT Pure strain of Saccharomyces cerevisae was used to ferment cassava pulp for 72 h with the aim of increasing the protein content of the cassava product. The mash obtained was processed to cassava flour, one of the forms in which cassava product is commonly consumed in Nigeria. The nutritional and toxicological potentials of the fungus fermented cassava flour were evaluated using rat bioassay. S. cerevisae fermented cassava flour (40%) fed to albino rat for 21 days had high feed conversion and digestibility (apparent and dry matter). Moreover, this level of cassava incorporation had no negative haematological (packed cell volume, red blood cell counts and white blood cell counts) effect. However, there was a significant (P 0.05) rise in the serum albumin and bilirubin. Further pathological investigation revealed that the spleen showed some dark red colouration while the liver had some necrotic lesion. The possible cause of this damage is the theme of further investigation in our laboratory.

*****

3. Saccharomyces cerevisiae, a potential pathogen towards grapevine, Vitis vinifera.

Sabine Gognies, Abdel Belarbi and Essaid Ait Barka.

UFR Sciences, Laboratoire de Microbiologie Generale et Moleculaire, Universite de Reims, 51687, Reims Cedex 2, France abdel.belarbi@univ-reims.fr

FEMS Microbiol.Ecol. 2001, 37 (2): 143-150

English

ABSTRACT Stresses applied to plants by pathogens such as fungi, bacteria, and viruses are well documented. However, to our knowledge, no study has focused on the effect of yeasts on plants. In this work the relationship between the growth of yeast, Saccharomyces cerevisiae, and its action on grapevine (Vitis vinifera L.) plantlets was studied. We observed that certain strains of S. cerevisiae could penetrate into the grapevine plants, bringing about a delay in the growth, or even causing the plantlets to die. We correlated this novel parasitic behavior of these strains of S. cerevisiae with their endopolygalacturonase activities and pseudohyphae formation. This study reports that the differences in behavior between the strains of S. cerevisiae are based on the filamentous forms, but that their pectolytic activities are required to invade grapevine tissues. The invasive process of the host plant has been confirmed histologically. Such yeast-plant interactions explain how S. cerevisiae may survive on grapevine throughout the years. The details of the parasitic relationship between S. cerevisiae and V. vinifera plantlets together with these parameters are discussed.

*****

4. **Nutritional and toxicological evaluation of yeast (Saccharomyces cerevisiae) biomass and a yeast protein concentrate.**

Glenys M. Caballero-Cordoba and Valdemiro C. Sgarbieri.

Centre of Food Chemistry and Applied Nutrition, Institute of Food Technology, AV Brasil 2880, CEP 13073-001, Campinas, SP, Brazil

J.Sci.Food Agric. 2000, 80 (3): 341-351

English

ABSTRACT Brewer's yeast was prepared by alkaline treatment for debittering, cell wall rupture and dehydration by spray drying. Yeast protein concentrate was prepared by centrifugation of the ruptured cell suspension, treatment of the supernatant with sodium perchlorate, precipitation of the protein at isoelectric pH (4.2) and neutralisation of the isoelectric protein to pH 6.5 with sodium hydroxide, prior to lyophylisation. Chemical characterisation was performed on the biomass and protein concentrate. Amino acid scores were 98.1 and 87.2% for the whole biomass and protein concentrate respectively, based on available lysine and compared with the FAO/WHO/UNU reference standard. The growth-promoting property of the yeast biomass protein was roughly 85% of casein and was significantly better than for the yeast protein concentrate. No difference in growth was found between 15 and 30% dietary protein for all three sources, ie casein, whole yeast biomass and yeast protein concentrate. When tested for subchronical toxicity at 15 and 30% protein concentration, no evidence of toxicity was found for the whole yeast biomass, compared with casein, after 45 and 90 days of feeding. Retarded growth and discrete liver steatosis were observed in rats fed the yeast protein concentrate at both dietary levels.

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5. **Pathogenicity of Saccharomyces cerevisiae in complement factor five-deficient mice.**

J. K. Byron, K. V. Clemons, J. H. McCusker, R. W. Davis and D. A. Stevens.

Department of Biology, Stanford University, California.

Infect.Immun. 1995, 63 (2): 478-485

English

ABSTRACT We have previously determined the relative virulence of isolates of Saccharomyces cerevisiae on the basis of differences in proliferation and resistance to clearance in CD-1 mice. These infections were not fatal. To further characterize S. cerevisiae pathogenesis, we studied a virulent clinical isolate, YJM128, and an avirulent nonclinical isolate, Y55, in C5-deficient mice. DBA/2N mice were infected intravenously with YJM128 or Y55, and temporal burdens of yeast cells in various organs were determined. After infection with 10(7) CFU, Y55 increased by 13-fold and YJM128 increased by 20-fold in the brain from day 0 to 3. In addition, YJM128 increased by 4-fold in the kidneys, whereas Y55 decreased by 16-fold. Both isolates declined in number in other organs. In all studies, 90% of mice infected with 10(7) CFU of YJM128 died between days 2 and 7, whereas no mice infected with equivalent numbers of Y55 died. No mice died after infection with 10(6) CFU of Y55 or YJM128. The importance of C5 was confirmed by studies using B10.D2/oSnJ (C5-) mice and their congenic C5+ counterparts. Again, the C5- mice were most susceptible to infection with S. cerevisiae, with 63% infected with YJM128 dying by day 7; no C5+ mice died. No Y55-infected mice died, and mean burdens in the brain at day 14 were sevenfold lower in C5+ mice than in C5- mice. Seven of 10 other S. cerevisiae isolates were also more virulent in DBA/2N than CD-1 mice, causing > or = 40% mortality. These data indicate that C5 is a critical factor in host resistance against S. cerevisiae infections and further confirm the pathogenic potential of some isolates of S. cerevisiae.

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6. **Colonization and pathogenicity of Saccharomyces cerevisiae, MC16, in mice and cynomolgus monkeys after oral and intravenous administration.**

K. Maejima, K. Shimoda, C. Morita, T. Fujiwara and T. Kitamura.

Jpn.J.Med.Sci.Biol. 1980, 33 (5): 271-276

English

ABSTRACT Saccharomyces cerevisiae, an ascomycetous yeast and a candidate for a host-vector system in recombinant DNA experiments, was examined for its pathogenicity and colonization in mice and cynomolgus monkeys as the models of the biological containment level. Adult mice given perorally with 5.5 or 2.4X10(7) cells of MC16 strain S. cerevisiae excluded them rapidly and no colonization of the cells in the abdominal organs, lymph nodes or gastrointestinal wall was demonstrated. No change in the fecal flora was observed. Cynomolgus monkeys after peroral administration of 4.9X10(7) or 7.8X10(8) cells showed a similar tendency of rapid exclusion and the lack of colonization. Cortisone acetate treatment had no significant effect. Intravenous administration of 3.9X10(7) yeast cells had no pathogenic effect and no viable yeast cell was detected in the blood. The biological containment level of S. cerevisiae was suggested to be not lower than that of E. coli K12 B1 level, and the possibility of achieving the B2 level was also suggested.

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7. **Un cas de blastomycose inveteree trans-missible au cobaye, due a un Saccharomyces pathogene (Saccharomyces jadini n. sp.).**

A. SARTORY, R. SARTORY, J. WEILL and J. MEYER.

COMPT REND ACAD SCI [PARIS] 1932, 194 (19): 1688-1690

Unavailable

ABSTRACT A yeast, Saccharomyces jadini, was isolated from an abscess in the sacral region, the cells of which were round, 4-5u, in diam., and which showed numerous asci with 2-4 ascospores; it did not liquefy gelatin or attack albumin, it coagulated milk without peptonization, and fermented glucose and maltose but not lactose or levulose. Intracardiac inoculation of guinea pigs caused the appearance of lymphatic nodules, hypertrophy of the spleen and liver, and abscesses in these organs and in the lungs. The urine reduced Fehling's solution. Death occurred in 5-7 mos. Retro-cultures were positive. The patient was treated by intradermic injections of $CuSO_4$, neoidipine, insulin and ultraviolet radiation. ABSTRACT AUTHORS: I. M. Korr

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Indirect indications of pathogenicity, primarily by genetic or other structural arguments. Publications on *Saccharomyces* isolated from clinical specimen are included in this list too.

8. **Stronger purifying selection against gene conversions in a pathogenic Saccharomyces cerevisiae strain.**

Benoit Page and Guy Drouin.

Departement de Biologie et Centre de Recherche Avancee en Genomique Environnementale, Universite d'Ottawa, 30 Marie Curie, Ottawa, ON K1N 6N5, Canada.

Genome 2012, 55 (12): 835-843

English

ABSTRACT Gene conversions most often have no selective impact, but some are selectively disadvantageous whereas others are selectively advantageous. Although gene conversions have been extensively studied in yeasts, very little is known about their selective impact in pathological yeasts. Here, we used the GENECONV software to compare the characteristics of candidate gene conversions found in a pathogenic strain (YJM789) and a nonpathogenic strain (S288c) of Saccharomyces cerevisiae. Interestingly, the pathogenic strain has fewer gene conversions when compared with the nonpathogenic strain. Of the 123 conversions we identified, 27 were identical or similar between the two strains, 62 were specific to the S288c strain, and 34 were specific to the YJM789 strain. Identical and similar conversions likely represent conversions that are under similar levels of purifying selection in both strains. The lower number of gene conversions in most gene families of the pathogenic strain is likely the result of higher purifying selection in this strain. In contrast, the higher number of conversions found in the YRF1 helicase gene family of the pathogenic strain could represent an example of adaptive gene conversions involved in maintaining its telomeres.

*****

9. **Genome-wide association analysis of clinical vs. nonclinical origin provides insights into Saccharomyces cerevisiae pathogenesis .**

L. A. H. Muller, J. E. Lucas, D. R. Georgianna and J. H. McCusker.

Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA.

Mol. Ecol. 2011, 20 (19): 4085-4097

English

ABSTRACT Because domesticated Saccharomyces cerevisiae strains have been used to produce fermented food and beverages for centuries without apparent health implications, S. cerevisiae has always been considered a Generally Recognized As Safe (GRAS) microorganism. However, the number of reported mucosal and systemic S. cerevisiae infections in the human population has increased and fatal infections have occurred even in relatively healthy individuals. In order to gain insight into the pathogenesis of S. cerevisiae and improve our understanding of the emergence of fungal pathogens, we performed a population-based genome-wide environmental association analysis of clinical vs. nonclinical origin in S. cerevisiae. Using tiling array-based, high-density genotypes of 44 clinical and 44 nonclinical S. cerevisiae strains from diverse geographical origins and source substrates, we identified several genetic loci associated with clinical background in S. cerevisiae. Associated polymorphisms within the coding sequences of VRP1, KIC1, SBE22 and PDR5, and the 5' upstream region of YGR146C indicate the importance of pseudohyphal formation, robust cell wall maintenance and cellular detoxification for S. cerevisiae pathogenesis, and constitute good candidates for follow-up verification of virulence and virulence-related factors underlying the pathogenicity of S. cerevisiae. .COPYRGT. 2011 Blackwell Publishing Ltd.

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10. **The Cytosolic Tail of the Golgi Apyrase Ynd1 Mediates E4orf4-Induced Toxicity in Saccharomyces cerevisiae.**

Karin Mittelman, Keren Ziv, Tsofnat Maoz and Tamar Kleinberger.

Weizmann Inst Sci, Dept Mol Genet, IL-76100 Rehovot, Israel tamark@tx.technion.ac.il

PLoS One 2010, 5 (11): Article No: e15539

English

ABSTRACT The adenovirus E4 open reading frame 4 (E4orf4) protein contributes to regulation of the progression of virus infection. When expressed individually, E4orf4 was shown to induce non-classical transformed cell-specific apoptosis in mammalian cells. At least some of the mechanisms underlying E4orf4-induced toxicity are conserved from yeast to mammals, including the requirement for an interaction of E4orf4 with protein phosphatase 2A (PP2A). A genetic screen in yeast revealed that the Golgi apyrase Ynd1 associates with E4orf4 and contributes to E4orf4-induced toxicity, independently of Ynd1 apyrase activity. Ynd1 and PP2A were shown to contribute additively to E4orf4-induced toxicity in yeast, and to interact genetically and physically. A mammalian orthologue of Ynd1 was shown to bind E4orf4 in mammalian cells, confirming the evolutionary conservation of this interaction. Here, we use mutation analysis to identify the cytosolic tail of Ynd1 as the protein domain required for mediation of the E4orf4 toxic signal and for the interaction with E4orf4. We also show that E4orf4 associates with cellular membranes in yeast and is localized at their cytoplasmic face. However, E4orf4 is membrane-associated even in the absence of Ynd1, suggesting that additional membrane proteins may mediate E4orf4 localization. Based on our results and on a previous report describing a collection of Ynd1 protein partners, we propose that the Ynd1 cytoplasmic

tail acts as a scaffold, interacting with a multi-protein complex, whose targeting by E4orf4 leads to cell death.

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11. **Analyses of the effects of Rck2p mutants on Pbs2pDD-induced toxicity in Saccharomyces cerevisiae identify a MAP kinase docking motif, and unexpected functional inactivation due to acidic substitution of T379.**

L. Jiang, S. Niu, K. L. Clines, D. J. Burke and T. W. Sturgill.

Department of Pharmacology, University of Virginia Health Sciences Center, PO Box 800735, Charlottesville, VA 22908, USA.

MGG 2004, 271 (2): 208-219

English

ABSTRACT Rck2p is a Ser/Thr kinase that binds to, and is activated by, Hog1p. Expression of the MAP kinase kinase Pbs2pDD from a GAL1-driven plasmid hyperactivates the HOG MAP kinase pathway, and leads to cessation of growth. This toxic effect is reduced by deletion of RCK2. We studied the structural and functional basis for the role of Rck2p in mediating the growth arrest phenotype associated with overexpression of Pbs2pDD. Rck2p kinase activity is required for the effect, because Rck2p(Delta487-610), as well as full-length Rck2p, is toxic with Pbs2pDD, but kinase-defective versions of either protein with a K201R mutation are not. Thus, the C-terminal portion of Rck2p is not required provided the protein is activated by removal of the autoinhibitory domain. Relief of inhibition in Rck2p normally requires phosphorylation by Hog1p, and Rck2p contains a putative MAP kinase docking site (TILQR589R590KKVQ) in its C-terminal segment. The Rck2p double mutant R589A/R590A expressed from a centromeric plasmid did not detectably bind Hog1p-GFP and was functionally inactive in mediating the toxic effect of Pbs2pDD, equivalent to an RCK2 deletion. However, overexpression of Rck2p R589A/R590A from a multicopy plasmid restored function. In contrast, RCK2-K201R acted as a multicopy suppressor of PBS2DD, markedly reducing its toxicity. This suppressor activity required the K201R mutation, and the effect was largely lost when the docking site was mutated, suggesting suppression by inhibition of Hog1p functions. We also studied the effect of replacing the predicted T379 and established S520 phosphorylation sites in Rck2p by glutamic acid. Surprisingly, the T379E mutant markedly reduced Pbs2pDD toxicity, and toxicity was only partially rescued by S520E. Rck2 T379E was sufficiently inactive in an rck2Delta strain to allow some cells to survive PBS2DD toxicity even when overexpressed. The significance of these findings for our understanding of Rck2p function is discussed.

*****

12. **Global network analysis of phenotypic effects: protein networks and toxicity modulation in Saccharomyces cerevisiae.**

R. Said Maya, J. Begley Thomas, V. Oppenheim Alan, A. Lauffenburger Douglas and D. Samson Leona.

Digital Signal Processing Group, Department of Electrical Engineering and Computer Science, and Biological Engineering Division and Center for Environmental Health Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

Proc.Natl.Acad.Sci.U.S.A. 2004, 101 (52): 18006-18011

English

ABSTRACT Using genome-wide information to understand holistically how cells function is a major challenge of the postgenomic era. Recent efforts to understand molecular pathway operation from a global perspective have lacked experimental data on phenotypic context, so insights concerning biologically relevant network characteristics of key genes or proteins have remained largely speculative. Here, we present a global network investigation of the genotype/phenotype data set we developed for the recovery of the yeast Saccharomyces cerevisiae from exposure to DNA-damaging agents, enabling explicit study of how protein-protein interaction network characteristics may be associated with phenotypic functional effects. We show that toxicity-modulating proteins have similar topological properties as essential proteins, suggesting that cells initiate highly coordinated responses to damage similar to those needed for vital cellular functions. We also identify toxicologically important protein complexes, pathways, and modules. These results have potential implications for understanding toxicity-modulating processes relevant to a number of human diseases, including cancer and aging.

*****

13. **Genetic characterization of pathogenic Saccharomyces cerevisiae isolates.**

J. H. McCusker, K. V. Clemons, D. A. Stevens and R. W. Davis.

Department of Biochemistry, Stanford University School of Medicine, California 94305.

Genetics 1994, 136 (4): 1261-1269

English

ABSTRACT Saccharomyces cerevisiae isolates from human patients have been genetically analyzed. Some of the characteristics of these isolates are very different from laboratory and industrial strains of S. cerevisiae and, for this reason, stringent genetic tests have been used to confirm their identity as S. cerevisiae. Most of these clinical isolates are able to grow at 42 degrees, a temperature that completely inhibits the growth of most other S. cerevisiae strains. This property can be considered a virulence trait and may help explain the presence of these isolates in human hosts. The ability to grow at 42 degrees is shown to be polygenic with primarily additive effects between loci. S. cerevisiae will be a useful model for the evolution and genetic analysis of fungal virulence and the study of polygenic traits.

*****

14. **IgE-binding components of baker's yeast (Saccharomyces cerevisiae) recognized by immunoblotting analysis. Simultaneous IgE binding to mannan and 46-48 kD allergens of Saccharomyces cerevisiae and Candida albicans.**

O. Kortekangas-Savolainen, K. Kalimo, K. Lammintausta and J. Savolainen.

Department of Dermatology, University of Turku, Finland.

Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 1993, 23 (3): 179-184

English

ABSTRACT The Saccharomyces cerevisiae allergens were characterized by IgE-immunoblotting with serum samples of 83 patients; 63 represented patients with atopic dermatitis with previous positive skin prick test or RAST for S. cerevisiae, seven patients with AD but negative test results and 13 were non-atopic controls. Disrupted whole body

extract of S. cerevisiae was used in the assays. From the patients tested 41 patients with atopic dermatitis appeared positive in IgE immunoblotting revealing 22 IgE stained bands. From these bands 10 represented intermediate allergens, and 12 minor allergens. The most frequent staining was obtained with the 48 kD band (39%). When the staining pattern of 45 kD and 48 kD bands and mannan was compared with Candida albicans allergens or purified baker's yeast enolase a simultaneous binding was seen with the 48 kD band of S. cerevisiae and the 46 kD band of C. albicans and enolase whereas the 45 kD band was neither associated with the 46 kD band of C. albicans nor purified enolase. High molecular weight staining was found in five samples. The staining pattern was associated with the mannose containing structures in parallel with C. albicans.

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15. **The pathogenicity of Saccharomyces and personal case reports [French summ.J. Original Title: I pathogonos drasis ton saccharomykiton kai perigrafi idion periptoseon [French summ.J.**

A. STYLIANEA.

Acta Microbiol.Hell. 1963, 8 (3/4): 6-107

Unavailable

ABSTRACT From a case of onychia and a case of gastroenteritis Saccharomyces cerevisiae was isolated.

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16. **Saccharomyces carlsbergenesis, possibly a pathogenic .**

S. REIERSOL and J. HOEL.

Acta Pathol.Microbiol.Scand. 1958, 44 (3): 313-318

English

XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

Killer toxins are produced by Saccharomyces strains in order to kill other microorganisms. Therfore these strains formally can be called toxic. Publications on that topic are listed here.

17. **Saccharomyces cerevisiae produced K2 toxin effects on microbial cells.**

(correspondence) Orentaite I., L. Zilinskiene, E. Serviene and R. Daugelavicius.

Vytautas Magnus University, Department of Biochemistry and Biotechnologies, Vileikos 8, Kaunas, LT-44404, Lithuania. irmaorentaite@gm ail.com; Nature Research Centre, Institute of Botany, Laboratory of Genetics, Akademijos 2, Vilnius, LT-08412, Lithuania. irmaorentaite@gmail.com; Vilnius Gediminas Technical University, Department of Chemistry and Bioengineering, Sauletekio 11, Vilnius, LT-10223, Lithuania. irmaorentaite @gmail.com

Biochimica et Biophysica Acta - Bioenergetics, . October 2012, 1817 (Supp 1): S158-S159

English

ABSTRACT Certain Saccharomyces cerevisiae strains produce killer toxins which kill sensitive cells. This phenomenon is important for strains to survive in natural and industrial populations. Toxins are able to kill the nonkiller and different type killer yeast belonging to the same or other genera while the producing cells remain immune to the action of its own or relative toxins. Depending on the differences of toxins' molecular features, killer effects and encoding genetic determinants there are three types of killer systems: K1, K2, and K28. Among those, the mechanism of action of K2 killer is the least understood. In order to describe the mechanism of K2 action it is necessary to analyse not only the intraspecial activity but also its effect on other microorganisms. It is known that some yeast killer strains have antibacterial activity but the performed microbiological tests are not sensitive enough to determine it. Instrumental studies carried out with susceptible yeasts and bacteria are more sensitive. The objective of our work was the evaluation of K2 killer toxins' impact on different microorganisms applying microbiological methods and performing bioluminescence and electrochemical analysis to characterise the energetical state of cells. It was shown that during the action K2 toxin decreases the level of ATP and the gradients of small molecules. Our study demonstrated that measurements of bioluminescence and electrochemical analysis are very useful methods for determination of the sensitivity of cells to toxin and the analysis of killing mechanisms.

*****

18. **Production and effect of killer toxin by Saccharomyces cerevisiae on sensitive yeast and fungal pathogens.**

(correspondence) Mohamudha Parveen R. and J. Ayesha Begum.

Department of Microbiology, Adhiparasakthi College of Arts and Science, G.B.Nagar, Kalavai, Vellore District- 632506, India. mahamudhaparveen@gmai l.com

Int. J. Pharm. Sc. Rev. Res. July - August 2010, 3 (1): 127-129

English

ABSTRACT Killer toxins are the proteinaceous toxins produced by some group of yeast designated- Killer yeasts. These toxins have activity against microorganisms other than yeast and the activity is readily detectable only when a suitable sensitive strain is tested. The aim of this study was extract the Killer toxin from yeast cells, to purify by thin layer chromatography and to find out the action of killer toxin on different fungal pathogens.

*****

19. **A new wine Saccharomyces cerevisiae killer toxin (Klus), encoded by a double-stranded rna virus, with broad antifungal activity is evolutionarily related to a chromosomal host gene.**

Nieves Rodriguez-Cousino, Matilde Maqueda, Jesus Ambrona, Emiliano Zamora, Rosa Esteban and Manuel Ramirez.

Departamento de Microbiologia (Antiguo Rectorado), Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain.

App. Environ. Microbiol. 2011, 77 (5): 1822-1832

English

ABSTRACT Wine Saccharomyces cerevisiae strains producing a new killer toxin (Klus)

were isolated. They killed all the previously known S. cerevisiae killer strains, in addition to other yeast species, including Kluyveromyces lactis and Candida albicans. The Klus phenotype is conferred by a medium-size double-stranded RNA (dsRNA) virus, Saccharomyces cerevisiae virus Mlus (ScV-Mlus), whose genome size ranged from 2.1 to 2.3 kb. ScV-Mlus depends on ScV-L-A for stable maintenance and replication. We cloned and sequenced Mlus. Its genome structure is similar to that of M1, M2, or M28 dsRNA, with a 5'-terminal coding region followed by two internal A-rich sequences and a 3'-terminal region without coding capacity. Mlus positive strands carry cis-acting signals at their 5' and 3' termini for transcription and replication similar to those of killer viruses. The open reading frame (ORF) at the 5' portion codes for a putative preprotoxin with an N-terminal secretion signal, potential Kex2p/Kexlp processing sites, and N-glycosylation sites. No sequence homology was found either between the Mlus dsRNA and M1, M2, or M28 dsRNA or between Klus and the K1, K2, or K28 toxin. The Klus amino acid sequence, however, showed a significant degree of conservation with that of the product of the host chromosomally encoded ORF YFR020W of unknown function, thus suggesting an evolutionary relationship.

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20. **Possible Involvement of Plasmids in the Expression of Killer Toxins of Saccharomyces cerevisiae.**

Sahar A. Alshalchi, Sijal W. Alrikabi and Ahmad Sahi.

Care of Alshalchi F, Stenhagsvagen 131, S-75260 Uppsala, Sweden

Journal of Pure and Applied Microbiology 2009, 3 (2): 453-456

English

ABSTRACT Four isolates of Saccharomyces cerevisiae were selected in this study for their high ability to produce killer toxin, to investigate possible correlation between their plasmids contents and killer activity Plasmid's profiles on agarose gel revealed presence of two plasmids in each of four isolates Yeast cells obtained after plasmid curing experiments at elevated temperature showed that cured isolates lost their killing capacity The plasmid cured yeast cells originated from three yeast isolates (Scs7, Scvi8 and Scf14) showed the possible correlation between plasmid curing and loss of killing ability, whereas isolate Scf4 retained the killing ability in absence of plasmids Moreover, the extracted plasmid samples were shown to be sensitive to RNase treatment These results might suggest that the genetic determinants for toxin production m three isolates (Scs7, Scvi8 and Scf14) of selected killer's yeasts of S cerevisiae are encoded by dsRNA plasmid(s) except one isolate (Scf4), in this isolate the killer protein may be mediated by chromosome

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21. **Research of toxin and plasmids of Saccharomyces cerevisiae.**

Y. Qin and D. Gao.

Department of Microbiology, Shandong University, Jinan 250100.

Wei Sheng Wu Hsueh Pao 2000, 40 (1): 105-107

Chinese

ABSTRACT Killer toxin from Saccharomyces cerevisiae SK was isolated by ultrafiltration of culture supernatants and purified by poly(ethylene glycol). The toxin

migrates as one single protein band on SDS-PAGE and its molecular weight is 15 kD. The SK toxin has the greatest lethal effect on the sensitive yeast strain in the lat-lag phase. Extraction and purification of killer heretity factor(dsRNA) from SK found that M-dsRNA plasmid and L-dsRNA plasmid have different molecular lengths being 1.7 kb and 4.0 kb.

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22. **Killer toxin of Saccharomyces cerevisiae Y500-4L active against Fleischmann and Itaiquara commercial brands of yeast.**

Giselle A. M. Soares and Helia H. Sato.

Departamento de Ciencia de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas - UNICAMP, Campinas, SP, Brazil

Rev.Microbiol. 1999, 30 (3): 253-257

English

ABSTRACT The strain Saccharomyces cerevisiae Y500-4L, previously selected from the must of alcohol producing plants and showing high fermentative and killer capacities, was characterized according to the interactions between the yeasts and examined for curing and detection of dsRNA plasmids, which code for the killer character. The killer yeast S. cerevisiae Y500-4L showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against the standard killer yeasts K2 (S. diastaticus NCYC 713), K4 (Candida glabrata NCYC 388) and K11 (Torulopsis glabrata ATCC 15126). However S. cerevisiae Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K8 (Hansenula anomala NCYC 435), K9 (Hansenula mrakii NCYC 500), K10 (Kluyveromyces drosophilarum NCYC 575) and K11 (Torulopsis glabrata ATCC 15126). No M-dsRNA plasmid was detected in the S. cerevisiae Y500-4L strain and these results suggest that the genetic basis for toxin production is encoded by chromosomal DNA. The strain S. cerevisiae Y500-4L was more resistant to the loss of the phenotype killer with cycloheximide and incubation at elevated temperatures (40degreeC) than the standard killer yeast S. cerevisiae K1.

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23. **Isolation and properties of a chromosome-dependent KHR killer toxin in Saccharomyces cerevisiae.**

K. Goto, T. Iwase, K. Kichise, K. Kitano, A. Totuka, T. Obata and S. Hara.

National Research Institute of Brewing, 2-6-30 Takinogawa, Kita-ku, Tokyo 114, Japan.

Agric.Biol.Chem. 1990, 54 (2): 505-509

English

ABSTRACT A strain of the yeast Saccharomyces cerevisiae coding for KHR on the chromosome secreted a toxin that kills sensitive yeasts. The transformants of multicopy vectors carrying the KHR gene could secrete 3-4-fold the killer toxin of the donor strain. This toxic substance was purified 80-fold in specific activity from the culture filtrate by gel filtration and hydrophobic column chromatography. The purified toxin gave a single protein band with molecular mass of 20 kDa on SDS-PAGE and had an isoelectric point of pH 5.3. The toxin had novel killer activity against Candida glabrata and S. cerevisiae, but did not affect bacteria, fungi, or other yeasts.

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24. **THE K1 TOXIN OF SACCHAROMYCES -CEREVISIAE KILLS SPHEROPLASTS OF MANY YEAST SPECIES.**

H. ZHU and H. BUSSEY.

DEP BIOL, MCGILL UNIV, 1205 AVE DR PENFIELD, MONTREAL, QUEBEC, CANADA H3A 1B1

Appl.Environ.Microbiol. 1989, 55 (8): 2105-2107

ENGLISH

ABSTRACT The Saccharomyces cerevisiae K1 toxin killed spheroplasts from the genera Candida, Kluyveromyces, and Schwanniomyces. Cells of these organisms were toxin insensitive. The toxin bound poorly to Kluyveromyces factis cells. In contrast, Candida albicans bound the toxin to an extent similar to that seen with S. cerevisiae. Thus, wall receptors can define toxin specificity and are necessary but not sufficient for toxin action on intact cells.

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25. **[Comparative genetics of yeasts. XXIII. Unusual inheritance of toxin formation in Saccharomyces paradoxus batschinskaia]. Sravnitel'naia genetika drozhzhei. Soobshchenie XXIII. Neobychainoe nasledovanie toksinoobrazovaniia u Saccharomyces paradoxus batschinskaia.**

G. I. Naumov.

Genetika 1985, 21 (11): 1794-1798

Russian

ABSTRACT The data about cytoplasmic control of toxin formation in Saccharomyces paradoxus CBS5829 are presented. A novel determinant (KIL-k3) is probably located in the mitochondrial genome. In other mutations, adenine deficiency results in suppression of toxin formation of the K3 type. A new killer plasmid (KIL-kx) was detected in Sacch. paradoxus VKM Y-2472.

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26. **Secretion of Saccharomyces cerevisiae killer toxin : processing of the glycosylated precursor.**

H. Bussey, D. Saville, D. Greene, D. J. Tipper and K. A. Bostian.

Mol.Cell.Biol. 1983, 3 (8): 1362-1370

English

ABSTRACT Killer toxin secretion was blocked at the restrictive temperature in Saccharomyces cerevisiae sec mutants with conditional defects in the S. cerevisiae secretory pathway leading to accumulation of endoplasmic reticulum (sec18), Golgi (sec7), or secretory vesicles (sec1). A 43,000-molecular-weight (43K) glycosylated protoxin was found by pulse-labeling in all sec mutants at the restrictive temperature. In sec18 the protoxin was stable after a chase; but in sec7 and sec1 the protoxin was unstable, and in sec1 11K toxin was detected in cell lysates. The chymotrypsin inhibitor tosyl-l-phenylalanyl chloromethyl ketone (TPCK) blocked toxin secretion in vivo in wild-type cells by inhibiting protoxin cleavage. The unstable protoxin in wild-type and in sec7

and sec1 cells at the restrictive temperature was stabilized by TPCK, suggesting that the protoxin cleavage was post-sec18 and was mediated by a TPCK-inhibitable protease. Protoxin glycosylation was inhibited by tunicamycin, and a 36K protoxin was detected in inhibited cells. This 36K protoxin was processed, but toxin secretion was reduced 10-fold. We examined two kex mutants defective in toxin secretion; both synthesized a 43K protoxin, which was stable in kex1 but unstable in kex2. Protoxin stability in kex1 kex2 double mutants indicated the order kex1 --> kex2 in the protoxin processing pathway. TPCK did not block protoxin instability in kex2 mutants. This suggested that the KEX1- and KEX2-dependent steps preceded the sec7 Golgi block. We attempted to localize the protoxin in S. cerevisiae cells. Use of an in vitro rabbit reticulocyte-dog pancreas microsomal membrane system indicated that protoxin synthesized in vitro could be inserted into and glycosylated by the microsomal membranes. This membrane-associated protoxin was protected from trypsin proteolysis. Pulse-chased cells or spheroplasts, with or without TPCK, failed to secrete protoxin. The protoxin may not be secreted into the lumen of the endoplasmic reticulum, but may remain membrane associated and may require endoproteolytic cleavage for toxin secretion.

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27. **Protein secretion in yeast: Two chromosomal mutants that oversecrete killer toxin in Saccharomyces cerevisiae.**

H. Bussey, O. Steinmetz and D. Saville.

Department of Biology, McGill University, Montreal, Que. H3A 1B1, Canada.

Curr.Genet. 1983, 7 (6): 449-456

English

28. **PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR AND INTRA CELLULAR KILLER TOXIN OF SACCHAROMYCES -CEREVISIAE STRAIN 28.**

P. PFEIFFER and F. RADLER.

INSTITUT FUER MIKROBIOLOGIE UND WEINFORSCHUNG DER JOHANNES GUTENBER-UNIVERSITAET MAINZ, POSTFACH, 3980, D-6500 MAINZ, FEDERAL REPUBLIC OF GERMANY

J.Gen.Microbiol. 1982, 128 (11): 2699-2706

ENGLISH

ABSTRACT The extracellular killer toxin of S. cerevisiae strain 28 was concentrated by ultrafiltration of culture supernatants and purified by ion-exchange chromatography. Polyacrylamide gradient gel electrophoresis in SDS [sodium dodecyl sulfate] indicated that the toxin is a glycoprotein with a MW of .apprx. 16,000. Amino acid analysis revealed that the killer toxin contains 111 amino acid residues, equivalent to a MW of 14,045; the ratio of protein to carbohydrate in the molecule is .apprx. 9 to 1. The isoelectric point of the killer toxin was pH 4.4-4.5. The toxin was unaffected by heating at 40.degree. C for 1 h and its maximum activity against sensitive yeast cells occurred at pH 5.0. Cell-free extracts prepared from well-washed cells of S. cervisiae strain 28 were toxic for sensitive yeasts. The toxin present in these extracts (intracellular toxin) was partially purified by ultrafiltration and ion-exchange chromatography. The isoelectric points of the extracellular and intracellular killer toxin were similar.

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29. **Radioimmunoassay for yeast killer toxin from Saccharomyces cerevisiae.**

F. A. Siddiqui and H. Bussey.

Can.J.Microbiol. 1981, 27 (8): 847-849

English

ABSTRACT A radioimmunoassay was developed for the K1 killer toxin from strain T158C/S14a of Saccharomyces cerevisiae. 125I-labeled toxin was made to a specific activity of 100 microCi/mg of protein (1 microCi = 37 kBq). Antibody to purified toxin was prepared in rabbits using toxin cross-linked to itself. These antibodies, partially purified by 50% ammonium sulfate precipitation and Sepharose CL-6B column chromatography, produced one precipitation band with killer toxin and bound 125I-labeled toxin in a radioimmunoassay. The antibody preparation also bound with the toxins from another K1 killer, A364A, and three chromosomal superkiller mutants derived from it.

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30. **PRIMARY EFFECTS OF YEAST SACCHAROMYCES -CEREVISIAE KILLER TOXIN .**

P. DE LA PENA, F. BARROS, S. GASCON, S. RAMOS and P. S. LAZO.

DEP INTERFAC BIOQUIM, UNIV OVIEDO, SPAIN

Biochem.Biophys.Res.Commun. 1980, 96 (2): 544-550

ENGLISH

ABSTRACT Killer toxin from S. cerevisiae binds to sensitive cells immediately after addition to the cells. However, 50% mortality was obtained only after 40 min. Although it is thought that a lag phase is required for the killer to exert its action, experiments show that the killer starts affecting the cell immediately after binding. Thus, shortly after addition, the toxin was able to inhibit the transport of L-[3H]leucine as well as that of protons which are cotransported with this amino acid or with histidine. Moreover, killer toxin inhibited the pumping of protons to the medium by cells which were actively metabolizing glucose. These effects were a function of the concentration of toxin used. The results suggest that killer toxin acts by affecting the electrochemical proton gradient across the plasma membrane of yeast.

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31. **Yeast killer toxin: purification and characterisation of the protein toxin from Saccharomyces cerevisiae.**

R. G. Palfree and H. Bussey.

Eur. J. Biochem. 1979, 93 (3): 487-493

English

ABSTRACT Killer toxin from killer strains of Saccharomyces cerevisiae was isolated from concentrates of extracellular medium by precipitation in poly(ethylene glycol) and chromatography through glyceryl-controlled-pore glass. The toxin migrated as a single protein band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. A molecular weight of 11470 was determined for the toxin protein from its electrophoretic mobility and amino acid composition. Gel filtration of the active toxin indicated that the 11,470-Mr monomer was the active unit. Electrophoretic comparison of extracellular concentrates

from a killer strain and an isogenic non-killer showed the presence of the toxin protein only in the killer-derived material. The activity of the toxin was most stable between pH 4.2 and 4.6. At 30 degrees C toxin from a superkiller strain was more stable than that from a normal killer.

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32. **PURIFICATION AND PROPERTIES OF THE YEAST KILLER TOXIN FROM SACCHAROMYCES -CEREVISIAE.**

R. G. E. PALFREE and H. BUSSEY.

Abs, Ann. Meeting Am. Soc. Microbiol. 1978, 78 104

Unavailable

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Because of the "probiotic" action of *Saccharomyces boulardii,* which can be considered as a safety aspect, publications on that subject are listed here.

33. **Effectiveness and safety of Saccharomyces boulardii for acute infectious diarrhea.**

Cagri Dinleyici Ener, Makbule Eren, Metehan Ozen, Abidin Yargic Zeynel and Yvan Vandenplas.

Eskisehir Osmangazi University, Department of Pediatric Infectious Disease and Intensive Care Unit, Eskisehir, Turkey. timboothtr@yahoo.com

Exp. Op.Biol. Ther. 2012, 12 (4): 395-410

English

ABSTRACT INTRODUCTION: Acute diarrhea continues to be a leading cause of morbidity, hospitalization and mortality worldwide and probiotics have been proposed as a complementary therapy in the treatment of acute diarrhea. Regarding the treatment of acute diarrhea, a few probiotics including Saccharomyces boulardii seem to be promising therapeutic agents. AREAS COVERED: We performed a systematic review and meta-analysis regarding the use of S. boulardii in the treatment of acute infectious diarrhea with relevant studies that searched with the PubMed, Embase, Scopus, Google Scholar, the Cochrane Controlled Trials Library, and the Cochrane Database of Systematic Reviews through October 2011. This review describes the effects of S. boulardii on the duration of diarrhea, the risk of diarrhea during the treatment (especially at the third day) and duration of hospitalization in patients with acute infectious diarrhea. This review also focused on the potential effects of S. boulardii for acute infectious diarrhea due to different etiological causes. EXPERT OPINION: S. boulardii significantly reduced the duration of diarrhea approximately 24 h and that of hospitalization approximately 20 h. S. boulardii shortened the initial phase of watery stools; mean number of stools started to decrease at day 2; moreover, a significant reduction was reported at days 3 and 4. This systematic review and meta-analysis of the efficacy of S. boulardii in the treatment of acute infectious diarrhea show that there is strong evidence that this probiotic has a clinically significant benefit, whatever the cause, including in developing countries. Therefore, with S. boulardii, the shortened duration of diarrhea and the reduction in hospital stay result in social and economic benefits.

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34. **Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders.**

Theodoros Kelesidis and Charalabos Pothoulakis.

Department of Medicine, Division of Infectious Diseases, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA.

Therapeutic advances in gastroenterology 2012, 5 (2): 111-125

English

ABSTRACT Several clinical trials and experimental studies strongly suggest a place for Saccharomyces boulardii as a biotherapeutic agent for the prevention and treatment of several gastrointestinal diseases. S. boulardii mediates responses resembling the protective effects of the normal healthy gut flora. The multiple mechanisms of action of S. boulardii and its properties may explain its efficacy and beneficial effects in acute and chronic gastrointestinal diseases that have been confirmed by clinical trials. Caution should be taken in patients with risk factors for adverse events. This review discusses the evidence for efficacy and safety of S. boulardii as a probiotic for the prevention and therapy of gastrointestinal disorders in humans.

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35. **Efficacy and safety of Saccharomyces boulardii in acute childhood diarrhea: a double blind randomised controlled trial.**

Musheer Riaz, Seema Alam, Abida Malik and S. Manazir Ali.

Department of Pediatrics, Jawaharlal Nehru Medical College, AMU, Aligarh, UP, India.

Ind. J. Ped. 2012, 79 (4): 478-482

English

ABSTRACT OBJECTIVE: To see the efficacy and safety of 250 mg of Saccharomyces boulardii twice daily for 5 d in acute childhood diarrhea. METHODS: Children aged between 3 mo and 59 mo with acute onset diarrhea (of less than 48 h) admitted in DTTU (diarrhea treatment and training unit) were included and those with clinical evidence of severe malnutrition, systemic infection, encephalopathy and/or convulsion, electrolyte imbalance, invasive diarrhea or previous use of any probiotics were excluded from the study. Those included randomly were given either a placebo or Saccharomyces boullardi (SB) in identical packets mixed with puffed rice powder. RESULTS: Mean post intervention duration of diarrhea was significantly (95% CI = -28.13 to -5.43) shorter in SB group (52.08 .+-. 24.57 h) as compared to placebo group (64.04 .+-. 30.43 h). The time of appearance of first semi formed stool in SB group (39.48 .+-. 23.09 h) was significantly (95% CI -25.4 to -3.87) shorter than the placebo group (54.13 .+-. 28.21 h). No statistically significant difference was found in rest of the parameters. CONCLUSIONS: There is initial evidence available that SB may have a therapeutic role in the management of acute childhood diarrhea.

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36. **Efficacy and safety of Saccharomyces boulardii in amebiasis-associated diarrhea in children.**

Senay Savas-Erdeve, Songul Gokay and Yildiz Dallar.

Department of Pediatrics, Ankara Training and Research Hospital, Ankara, Turkey.

Turk.J.Pediatr. 2009, 51 (3): 220-224

English

ABSTRACT The efficacy and safety of adding Saccharomyces boulardii to antibiotic treatment for amebiasis-associated acute diarrhea in children were assessed in this study. Forty-five children in Group I received only metronidazole per oral for 10 days while 40 patients in Group II received S. boulardii in addition to the same medication. The major outcomes investigated were duration of acute and bloody diarrhea, frequency and consistency of stools, resolution time of the symptoms, and the tolerance and side effects of the treatment regimens. The median duration of acute diarrhea was 5 (1-10) days in Group I and 4.5 (1-10) days in Group II (p=0.965). The median number of stools on follow-up and duration of bloody diarrhea, fever, abdominal pain and vomiting were similar in the two groups. S. boulardii was well tolerated by the children and no side effects were recorded. Addition of S. boulardii to antibiotic treatment of amebiasis-associated acute diarrhea in children does not seem to be more effective than metronidazole treatment alone.

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37. **Efficacy and safety of Saccharomyces boulardii in the 14-day triple anti-Helicobacter pylori therapy: a prospective randomized placebo-controlled double-blind study.**

Mehmet Cindoruk, Gulbanu Erkan, Tarkan Karakan, Ayse Dursun and Selahattin Unal.

Department of Gastroenterology, School of Medicine, Gazi University, Ankara, Turkey.

Helicobacter 2007, 12 (4): 309-316

English

ABSTRACT BACKGROUND: Recent studies indicate a potential role of Saccharomyces boulardii in the prevention of Helicobacter pylori treatment-related side-effects and also in improvement of eradication rate. Our aim is to investigate the efficacy and safety of S. boulardii in the prevention of side-effects related to H. pylori eradication. The secondary aim of the study was to define the effect of S. boulardii on the eradication success of anti-H. pylori therapy. MATERIALS AND METHODS: One hundred and twenty-four patients with H. pylori infection (male/female: 44/80, mean age: 48 +/- 14.25 year) receiving 14 days of triple therapy (clarithromycin 500 mg b.i.d., amoxicillin 1000 mg b.i.d., and lansoprazole 30 mg b.i.d.) were randomly assigned to S. boulardii or placebo. Dyspeptic symptoms were recorded by using modified Glasgow Dyspepsia Questionnaire (GDQ). Side-effect profile and tolerability were assessed using a symptom-based questionnaire. H. pylori status was rechecked after 6 weeks after completion of eradication therapy. RESULTS: H. pylori eradication rate, although higher in the treatment group, was statistically similar in treatment and control groups: 71% (44/62) versus 59.7% (37/62), respectively ($p > .05$). Nine (14.5%) patients in the treatment group and 19 (30.6%) patients in the placebo group experienced diarrhea ($p < .05$). Epigastric discomfort was more frequent in the control group [9 (14.5%) versus 27 (43.5%), respectively ($p < .01$)]. Diffuse abdominal pain, abdominal gas, taste disturbance, urticaria, nausea symptoms were similar in both groups. GDQ scores after treatment were significantly better for treatment group (mean +/- SD, range: 1.38 +/- 1.25 (0-5) vs. 2.22 +/- 1.44 (0-6), respectively; $p < .01$). CONCLUSION: S. boulardii improved anti-H. pylori antibiotherapy-associated diarrhea, epigastric discomfort, and treatment tolerability. In addition, S. boulardii supplement decreased post-treatment dyspepsia symptoms independent of H. pylori status. However, S. boulardii had no

significant affect on the rate of H. pylori eradication.

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38. **Efficacy and safety of Saccharomyces boulardii in prevention of antibiotic-associated diarrhoea due to Helicobacterpylori eradication.**

Guney Duman Deniz, Serhat Bor, Omer Ozutemiz, Tulin Sahin, Dilek Oguz, Fahri Istan, Tumer Vural, Macit Sandkci, Fatih Isksal, Ilkay Simsek, Mujde Soyturk, Serap Arslan, Bulent Sivri, Irfan Soykan, Aysegul Temizkan, Fatih Bessk, Sabahattin Kaymakoglu and Cem Kalayc.

Department of Gastroenterology, Marmara University, School of Medicine, Istanbul, Turkey.

Eur.J.Gastroenterol.Hepatol. 2005, 17 (12): 1357-1361

English

ABSTRACT BACKGROUND AND AIM: Antibiotic-associated diarrhoea may develop during or following Helicobacter pylori eradication. We aimed to evaluate the efficacy and safety of Saccharomyces boulardii in preventing antibiotic-associated diarrhoea in patients receiving antibiotics for H. pylori eradication. METHODS: In a multicentre prospective clinical trial, patients with peptic ulcer disease or non-ulcer dyspepsia were enrolled to receive clarithromycin, amoxicillin and omeprazole for H. pylori eradication for 14 days. These patients were then randomized to receive either S. boulardii 500 mg twice daily (treatment group) or no treatment (control group). The primary outcome measure was the development of diarrhoea during (treatment period) or within 4 weeks after treatment (follow-up period). RESULTS: Of the 389 patients that were enrolled, 376 completed the study. Within the treatment period, diarrhoea developed in 5.9% of patients in the treatment group and in 11.5% of patients in the control group (P = 0.049); and in the follow-up period, diarrhoea developed in 1.0% of patients in the treatment group and in 3.8% of patients in the control group (P = 0.09). Overall diarrhoea rates throughout the whole study period were 6.9% in the treatment group and 15.6% in the control group (P = 0.007). No significant difference was observed between the treatment and control groups in terms of adverse events. CONCLUSION: S. boulardii is an effective and safe treatment for prevention of antibiotic-associated diarrhoea when given concomitantly to patients receiving H. pylori eradication.

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39. **Meta-analysis: non- pathogenic yeast Saccharomyces boulardii in the prevention of antibiotic-associated diarrhoea.**

H. Szajewska and J. Mrukowicz.

Department of Pediatric Gastroenterology and Nutrition, The Medical University of Warsaw, Warsaw, Poland. hania@ipgate.pl

Aliment.Pharmacol.Ther. 2005, 22 (5): 365-372

English

ABSTRACT BACKGROUND: Antibiotic-associated diarrhoea occurs in up to 30% of patients who receive antibiotics but can be prevented with probiotics. AIM: To systematically evaluate the effectiveness of Saccharomyces boulardii in preventing antibiotic-associated diarrhoea in children and adults. METHODS: Using medical subject headings and free-language terms, the following electronic databases were searched for

studies relevant to antibiotic-associated diarrhoea and S. boulardii: MEDLINE, EMBASE, CINAHL and The Cochrane Library. Additional sources were obtained from references in reviewed articles. Only randomized-controlled trials were considered for study inclusion. RESULTS: Of 16 potentially relevant clinical trials identified, five randomized-controlled trials (1076 participants) met the inclusion criteria for this systematic review. Treatment with S. boulardii compared with placebo reduced the risk of antibiotic-associated diarrhoea from 17.2% to 6.7% (RR: 0.43; 95% CI: 0.23-0.78; random effect model). The number needed to treat to prevent one case of antibiotic-associated diarrhoea was 10 (95% CI: 7-16). No side-effects were reported. CONCLUSIONS: A meta-analysis of data from five randomized-controlled trials showed that S. boulardii is moderately effective in preventing antibiotic-associated diarrhoea in children and adults treated with antibiotics for any reason (mainly respiratory tract infections). For every 10 patients receiving daily S. boulardii with antibiotics, one fewer will develop antibiotic-associated diarrhoea.

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40. **Experimental effects of Saccharomyces boulardii on diarrheal pathogens .**

Dorota Czerucka and Patrick Rampal.

Laboratoire de gastroenterologie et nutrition, universite de Nice-Sophia-Antipolis, faculte de medecine, 28 avenue de Valombrose, 06107 Nice cedex 2, France. czerucka@unice.fr

Microbes Infect. 2002, 4 (7): 733-739

English

ABSTRACT Saccharomyces boulardii is a selected strain of yeast that may have applications in the prevention and treatment of intestinal infections. The animal models and in vitro studies developed to elucidate the mechanisms of this protection are reviewed and discussed.

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All 40 selected papers can be viewed and obtained full-text via the RefShare link to RefWorks.

RefShare:
http://www.refworks.com/refshare2?site=042081168923600000/RWWS4A246139/yeast-safety

Appendix.

Long list of all retrieved publications:

L7 ANSWER 1 OF 184 MEDLINE ® on STN DUPLICATE 1
AN 2013186405 MEDLINE (IN-PROCESS) Full-text
DN PubMed ID: 23206402
TI Induction of protective immune responses against challenge of Actinobacillus pleuropneumoniae by oral administration with Saccharomyces cerevisiae expressing Apx toxins in pigs.
AU Shin Min-Kyoung; Kang Mi Lan; Jung Myung Hwan; Cha Seung-Bin; Lee Won-Jung; Kim Jung-Mi; Kim Dae-Hyuk; Yoo Han Sang
CS Department of Infectious Disease, College of Veterinary Medicine and Brain Korea 21 Program for Veterinary Science, Seoul National University, Seoul, Republic of Korea.
SO Veterinary immunology and immunopathology, (2013 Jan 15) Vol. 151, No. 1-2, pp. 132-9. Electronic Publication Date: 9 Nov 2012
Journal code: 8002006. E-ISSN: 1873-2534. L-ISSN: 0165-2427.
DOI 10.1016/j.vetimm.2012.11.003
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS NONMEDLINE; IN-PROCESS; NONINDEXED; Priority Journals
ED Entered STN: 5 Dec 2012
Last Updated on STN: 9 Feb 2013
AB Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia, a highly contagious endemic disease of pigs worldwide, inducing significant economic losses worldwide. Apx toxins, which are correlated with the virulence of A. pleuropneumoniae, were expressed in Saccharomyces cerevisiae and its possible use as an oral vaccine has been confirmed in our previous studies using a murine model. The present study was undertaken to test the hypothesis that oral immunization using S. cerevisiae expressing either ApxI or ApxII could protect pigs against A. pleuropneumoniae as an effective way of inducing both mucosal and systemic immune responses. The surface-displayed ApxIIA#5 expressing S. cerevisiae was selected as an oral vaccine candidate by finding on induction of higher immune responses in mice after oral vaccination. The surface-displayed ApxIIA#5 expressing S. cerevisiae and the ApxIA expressing S. cerevisiae were developed to serve as an oral vaccine in pigs. The vaccinated pigs showed higher specific IgG- and IgA-related antibody activities than the non-treated control and vector control pigs. Additionally, the induced immune responses were found to protect pigs infected with A. pleuropneumoniae according to the analysis of clinical signs and the gross and microscopic pulmonary lesions. These results suggested that the surface-displayed ApxIIA#5 and ApxIA in S. cerevisiae might be a potential oral vaccine to protect pigs against porcine pleuropneumonia. Thus the present study is expected to contribute to the development of a live oral vaccine against porcine pleuropneumonia as an alternative to current conventional vaccines. Copyright .COPYRGT. 2012 Elsevier B.V. All rights reserved.

L7 ANSWER 2 OF 184 MEDLINE ® on STN DUPLICATE 2
AN 2013017880 MEDLINE Full-text
DN PubMed ID: 23028482
TI Cch1p mediates Ca2+ influx to protect Saccharomyces cerevisiae against eugenol toxicity.
AU Roberts Stephen K; McAinsh Martin; Widdicks Lisa
CS Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, United Kingdom.
s.k.roberts@lancaster.ac.uk
SO PloS one, (2012) Vol. 7, No. 9, pp. e43989. Electronic Publication Date: 13 Sep 2012
Journal code: 101285081. E-ISSN: 1932-6203. L-ISSN: 1932-6203.
Report No.: NLM-PMC3441571.
DOI 10.1371/journal.pone.0043989
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 201303
ED Entered STN: 4 Oct 2012
Last Updated on STN: 20 Mar 2013
Entered Medline: 19 Mar 2013
REM.CNT 30 There are 30 cited references available in MEDLINE for this document.
AB Eugenol has antifungal activity and is recognised as having therapeutic potential. However, little is known of the cellular basis of its antifungal activity and a better understanding of eugenol tolerance should lead to better exploitation of eugenol in antifungal therapies. The model yeast, Saccharomyces cerevisiae, expressing apoaequorin was used to show that eugenol induces cytosolic Ca(2+) elevations. We investigated the eugenol Ca(2+) signature in further detail and show that exponentially growing cells exhibit Ca(2+) elevation resulting exclusively from the influx of Ca(2+) across the plasma membrane whereas in stationary growth phase cells Ca(2+) influx from intracellular and extracellular sources contribute to the eugenol-induced Ca(2+) elevation. Ca(2+) channel deletion yeast mutants were used to identify the pathways mediating Ca(2+) influx; intracellular Ca(2+) release was mediated by the vacuolar Ca(2+) channel, Yvc1p, whereas the Ca(2+) influx across the plasma membrane could be resolved into Cch1p-dependent and Cch1p-independent pathways. We show that the growth of yeast devoid the plasma membrane Ca(2+) channel, Cch1p, was hypersensitive to eugenol and that this correlated with reduced Ca(2+) elevations. Taken together, these results indicate that a cch1p-mediated Ca(2+) influx is part of an intracellular signal which protects against eugenol toxicity. This study provides fresh insight into the mechanisms employed by fungi to tolerate eugenol toxicity which should lead to better exploitation of eugenol in antifungal therapies.

L7 ANSWER 3 OF 184 MEDLINE ® on STN DUPLICATE 3
AN 2013188376 MEDLINE (IN-PROCESS) Full-text
DN PubMed ID: 23208446
TI Identification of deubiquitinating enzymes involved in methylmercury toxicity in Saccharomyces cerevisiae.
AU Hwang Gi-Wook; Kimura Yukiyoshi; Takahashi Tsutomu; Lee Jin-Yong; Naganuma Akira

CS Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Miyagi, Japan.
SO The Journal of toxicological sciences, (2012) Vol. 37, No. 6, pp. 1287-90. Journal code: 7805798. E-ISSN: 1880-3989. L-ISSN: 0388-1350.
CY Japan
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS NONMEDLINE; IN-PROCESS; NONINDEXED; Priority Journals
ED Entered STN: 5 Dec 2012
Last Updated on STN: 23 Jan 2013
AB Deubiquitinating enzymes that influence the methylmercury susceptibility of yeast cells were investigated. Deficiency of Ubp2, Ubp4, Ubp 6, or Ubp14 conferred methylmercury resistance on cells, while deficiency of Ubp7, Ubp13, or Ubp15 conferred high methylmercury susceptibility. Of these enzymes, deficiency of Ubp4 and Ubp6 was associated with particularly high methylmercury resistance. Yeast cells treated with a proteasome inhibitor showed methylmercury resistance due to Ubp4 deficiency, but not due to Ubp6 deficiency. Thus, the enhanced methylmercury toxicity associated with Ubp6 expression requires proteasomal activity, suggesting that Ubp4 and Ubp6 contribute to enhanced methylmercury toxicity through different mechanisms.

L7 ANSWER 4 OF 184 MEDLINE ® on STN DUPLICATE 4
AN 2012626955 MEDLINE Full-text
DN PubMed ID: 22580913
TI Adhesion to the yeast cell surface as a mechanism for trapping pathogenic bacteria by Saccharomyces probiotics.
AU Tiago F C P; Martins F S; Souza E L S; Pimenta P F P; Araujo H R C; Castro I M; Brandao R L; Nicoli Jacques R
CS Departamento de Microbiologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.
SO Journal of medical microbiology, (2012 Sep) Vol. 61, No. Pt 9, pp. 1194-207. Electronic Publication Date: 11 May 2012
Journal code: 0224131. E-ISSN: 1473-5644. L-ISSN: 0022-2615.
DOI 10.1099/jmm.0.042283-0
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201210
ED Entered STN: 15 May 2012
Last Updated on STN: 30 Oct 2012
Entered Medline: 29 Oct 2012
AB Recently, much attention has been given to the use of probiotics as an adjuvant for the prevention or treatment of gastrointestinal pathology. The great advantage of therapy with probiotics is that they have few side effects such as selection of resistant bacteria or disturbance of the intestinal microbiota, which occur when antibiotics are used. Adhesion of pathogenic bacteria onto the surface of probiotics instead of onto intestinal receptors could explain part of the probiotic effect. Thus, this study evaluated the adhesion of pathogenic bacteria onto the cell wall of Saccharomyces boulardii and Saccharomyces cerevisiae strains UFMG 905, W303 and BY4741. To understand the mechanism of adhesion of pathogens to yeast, cell-wall mutants of the parental strain of Saccharomyces cerevisiae BY4741 were used because of the difficulty of mutating polyploid yeast, as is the case for Saccharomyces cerevisiae and Saccharomyces boulardii. The tests of adhesion showed that, among 11 enteropathogenic bacteria tested, only Escherichia coli, Salmonella Typhimurium and Salmonella Typhi adhered to the surface of Saccharomyces boulardii, Saccharomyces cerevisiae UFMG 905 and Saccharomyces cerevisiae BY4741. The presence of mannose, and to some extent bile salts, inhibited this adhesion, which was not dependent on yeast viability. Among 44 cell-wall mutants of Saccharomyces cerevisiae BY4741, five lost the ability to fix the bacteria. Electron microscopy showed that the phenomenon of yeast-bacteria adhesion occurred both in vitro and in vivo (in the digestive tract of dixenic mice). In conclusion, some pathogenic bacteria were captured on the surface of Saccharomyces boulardii, Saccharomyces cerevisiae UFMG 905 and Saccharomyces cerevisiae BY4741, thus preventing their adhesion to specific receptors on the intestinal epithelium and their subsequent invasion of the host.

L7 ANSWER 5 OF 184 MEDLINE ® on STN DUPLICATE 5
AN 2013212921 MEDLINE (IN-PROCESS) Full-text
DN PubMed ID: 23231602
TI Stronger purifying selection against gene conversions in a pathogenic Saccharomyces cerevisiae strain.
AU Page Benoit; Drouin Guy
CS Departement de Biologie et Centre de Recherche Avancee en Genomique Environnementale, Universite d'Ottawa, 30 Marie Curie, Ottawa, ON K1N 6N5, Canada.
SO Genome / National Research Council Canada = Genome / Conseil national de recherches Canada, (2012 Dec) Vol. 55, No. 12, pp. 835-43. Electronic Publication Date: 19 Nov 2012
Journal code: 8704546. E-ISSN: 1480-3321. L-ISSN: 0831-2796.
DOI 10.1139/gen-2012-0098
CY Canada
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS NONMEDLINE; IN-PROCESS; NONINDEXED; Priority Journals
ED Entered STN: 14 Dec 2012
Last Updated on STN: 30 Jan 2013
AB Gene conversions most often have no selective impact, but some are selectively disadvantageous whereas others are selectively advantageous. Although gene conversions have been extensively studied in yeasts, very little is known about their selective impact in pathological yeasts. Here, we used the GENECONV software to compare the characteristics of candidate gene conversions found in a pathogenic strain (YJM789) and a nonpathogenic strain (S288c) of Saccharomyces cerevisiae. Interestingly, the pathogenic strain has fewer gene conversions when compared with the nonpathogenic strain. Of the 123 conversions we identified, 27 were identical or similar between the two strains, 62 were specific to the S288c strain, and 34 were specific to the YJM789 strain. Identical and similar conversions likely represent conversions that are under similar levels of purifying selection in both strains. The lower number of gene conversions in most gene families of the pathogenic strain is likely the result of higher purifying selection in this strain. In contrast, the higher number of conversions found in the YRF1 helicase gene family of the pathogenic strain could represent an example of adaptive gene conversions involved in maintaining its telomeres.

L7 ANSWER 6 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on

STN
AN 2012:690935 BIOSIS Full-text
DN PREV201200690935
TI Acute and Subacute Toxicity of Recombinant Saccharomyces cerevisiae Expressing Salmon Calcitonin.
AU Wang Peng; Jiang Yong; Tian Jingyun; Ma Jian; Zhang Xuecheng; Lu Xingbo [Reprint Author]
CS Shandong Acad Agr Sci, Jinan 250100, Peoples R China luxb99@sina.com
SO Chinese Journal of Applied and Environmental Biology, (AUG 25 2012) Vol. 18, No. 4, pp. 630-634. ISSN: 1006-687X.
DT Article
LA Chinese
ED Entered STN: 31 Oct 2012
Last Updated on STN: 20 Mar 2013
AB Oral administration recombinant Saccharomyces cerevisiae expressing salmon calcitonin made a new method for salmon calcitonin delivery system. Transgenic S. cerevisiae was evaluated for its acute (7 days) and subacute toxicity (8 weeks) by the oral route in KM mouse and Wistar rats respectively. In the acute toxicity test, every group did not produce any hazardous symptoms or deaths (LD50> 10 000 mg/kg). The subacute treatment with transgenic S. cerevisiae (2.0 and 0.5 g/kg) failed to change body weight gain, food and water consumption. Organ/weight ratios, haematological and biochemical indices analysis showed no significant differences (P > 0.05) in any of the parameters examined. There was no pathological change observed in slice. These results suggested that recombinant S. cerevisiae expressing salmon calcitonin had no negative influences on the tested animal. It was safe and nontoxic. Fig 3, Tab 3, Ref 16

L7 ANSWER 7 OF 184 MEDLINE ® on STN DUPLICATE 6
AN 2012364955 MEDLINE Full-text
DN PubMed ID: 21997865
TI Efficacy and safety of Saccharomyces boulardii in acute childhood diarrhea: a double blind randomised controlled trial.
AU Riaz Musheer; Alam Seema; Malik Abida; Ali S Manazir
CS Department of Pediatrics, Jawaharlal Nehru Medical College, AMU, Aligarh, UP, India.
SO Indian journal of pediatrics, (2012 Apr) Vol. 79, No. 4, pp. 478-82. Electronic Publication Date: 14 Oct 2011
Journal code: 0417442. E-ISSN: 0973-7693. L-ISSN: 0019-5456.
DOI 10.1007/s12098-011-0573-z
CY India
DT Journal; Article; (JOURNAL ARTICLE)
(RANDOMIZED CONTROLLED TRIAL)
(CLINICAL TRIAL)
LA English
FS MEDLINE; Priority Journals
EM 201208
ED Entered STN: 19 Mar 2012
Last Updated on STN: 1 Sep 2012
Entered Medline: 31 Aug 2012
AB OBJECTIVE: To see the efficacy and safety of 250 mg of Saccharomyces boulardii twice daily for 5 d in acute childhood diarrhea.

METHODS: Children aged between 3 mo and 59 mo with acute onset diarrhea (of less than 48 h) admitted in DTTU (diarrhea treatment and training unit) were included and those with clinical evidence of severe malnutrition, systemic infection, encephalopathy and/or convulsion, electrolyte imbalance, invasive diarrhea or previous use of any probiotics were excluded from the study. Those included randomly were given either a placebo or Saccharomyces boullardi (SB) in identical packets mixed with puffed rice powder.

RESULTS: Mean post intervention duration of diarrhea was significantly (95% CI = -28.13 to -5.43) shorter in SB group (52.08 ± 24.57 h) as compared to placebo group (64.04 ± 30.43 h). The time of appearance of first semi formed stool in SB group (39.48 ± 23.09 h) was significantly (95% CI -25.4 to -3.87) shorter than the placebo group (54.13 ± 28.21 h). No statistically significant difference was found in rest of the parameters.

CONCLUSIONS: There is initial evidence available that SB may have a therapeutic role in the management of acute childhood diarrhea.

L7 ANSWER 8 OF 184 MEDLINE ® on STN DUPLICATE 7
AN 2012224553 MEDLINE Full-text
DN PubMed ID: 22281500
TI Phosphate disruption and metal toxicity in Saccharomyces cerevisiae: effects of RAD23 and the histone chaperone HPC2.
AU Rosenfeld Leah; Culotta Valeria C
CS Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA.
NC ES 007308 (United States NIEHS NIH HHS)
ES 08996 (United States NIEHS NIH HHS)
P30 ES003819 (United States NIEHS NIH HHS)
R01 ES008996-15 (United States NIEHS NIH HHS)
SO Biochemical and biophysical research communications, (2012 Feb 10) Vol. 418, No. 2, pp. 414-9. Electronic Publication Date: 18 Jan 2012
Journal code: 0372516. E-ISSN: 1090-2104. L-ISSN: 0006-291X.
Report No.: NLM-NIHMS350788; NLM-PMC3465668.
DOI 10.1016/j.bbrc.2012.01.047
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
LA English
FS MEDLINE; Priority Journals
EM 201204
ED Entered STN: 14 Feb 2012
Last Updated on STN: 10 Apr 2012

Entered Medline: 9 Apr 2012

AB In cells, there exists a delicate balance between accumulation of charged metal cations and abundant anionic complexes such as phosphate. When phosphate metabolism is disrupted, cell-wide spread disturbances in metal homeostasis may ensue. The best example is a yeast pho80 mutant that hyperaccumulates phosphate and as result, also hyperaccumulates metal cations from the environment and shows exquisite sensitive to toxicity from metals such as manganese. In this study, we sought to identify genes that when over-expressed would suppress the manganese toxicity of pho80 mutants. Two classes of suppressors were isolated, including the histone chaperones SPT16 and HPC2, and RAD23, a well-conserved protein involved in DNA repair and proteosomal degradation. The histone chaperone gene HPC2 reversed the elevated manganese and phosphate of pho80 mutants by specifically repressing PHO84, encoding a metal-phosphate transporter. RAD23 also reduced manganese toxicity by lowering manganese levels, but RAD23 did not alter phosphate nor repress PHO84. We observed that the RAD23-reversal of manganese toxicity reflected its role in protein quality control, not DNA repair. Our studies are consistent with a model in which Rad23p partners with the deglycosylating enzyme Png1p to reduce manganese toxicity through proteosomal degradation of glycosylated substrate(s).

L7 ANSWER 9 OF 184 MEDLINE ® on STN DUPLICATE 8
AN 2012309068 MEDLINE Full-text
DN PubMed ID: 22335323
TI Effectiveness and safety of Saccharomyces boulardii for acute infectious diarrhea.
AU Dinleyici Ener Cagri; Eren Makbule; Ozen Metehan; Yargic Zeynel Abidin; Vandenplas Yvan
CS Eskisehir Osmangazi University, Department of Pediatric Infectious Disease and Intensive Care Unit, Eskisehir, Turkey. timboothtr@yahoo.com
SO Expert opinion on biological therapy, (2012 Apr) Vol. 12, No. 4, pp. 395-410. Electronic Publication Date: 16 Feb 2012
Journal code: 101125414. E-ISSN: 1744-7682. L-ISSN: 1471-2598.
DOI 10.1517/14712598.2012.664129
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA English
FS MEDLINE; Priority Journals
EM 201207
ED Entered STN: 15 Mar 2012
Last Updated on STN: 20 Jul 2012
Entered Medline: 19 Jul 2012
OSC.G 2 There are 2 MEDLINE records that cite this record

AB INTRODUCTION: Acute diarrhea continues to be a leading cause of morbidity, hospitalization and mortality worldwide and probiotics have been proposed as a complementary therapy in the treatment of acute diarrhea. Regarding the treatment of acute diarrhea, a few probiotics including Saccharomyces boulardii seem to be promising therapeutic agents. AREAS COVERED: We performed a systematic review and meta-analysis regarding the use of S. boulardii in the treatment of acute infectious diarrhea with relevant studies that searched with the PubMed, Embase, Scopus, Google Scholar, the Cochrane Controlled Trials Library, and the Cochrane Database of Systematic Reviews through October 2011. This review describes the effects of S. boulardii on the duration of diarrhea, the risk of diarrhea during the treatment (especially at the third day) and duration of hospitalization in patients with acute infectious diarrhea. This review also focused on the potential effects of S. boulardii for acute infectious diarrhea due to different etiological causes. EXPERT OPINION: S. boulardii significantly reduced the duration of diarrhea approximately 24 h and that of hospitalization approximately 20 h. S. boulardii shortened the initial phase of watery stools; mean number of stools started to decrease at day 2; moreover, a significant reduction was reported at days 3 and 4. This systematic review and meta-analysis of the efficacy of S. boulardii in the treatment of acute infectious diarrhea show that there is strong evidence that this probiotic has a clinically significant benefit, whatever the cause, including in developing countries. Therefore, with S. boulardii, the shortened duration of diarrhea and the reduction in hospital stay result in social and economic benefits.

L7 ANSWER 10 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 0050846652 EMBASE Full-text
TI Saccharomyces cerevisiae produced K2 toxin effects on microbial cells.
AU Orentaite, I. (correspondence); Zilinskiene, L.; Serviene, E.; Daugelavicius, R.
CS Vytautas Magnus University, Department of Biochemistry and Biotechnologies, Vileikos 8, Kaunas, LT-44404, Lithuania. irmaorentaite@gmail.com
AU Orentaite, I. (correspondence); Zilinskiene, L.; Serviene, E.; Daugelavicius, R.
CS Nature Research Centre, Institute of Botany, Laboratory of Genetics, Akademijos 2, Vilnius, LT-08412, Lithuania. irmaorentaite@gmail.com
AU Orentaite, I. (correspondence); Zilinskiene, L.; Serviene, E.; Daugelavicius, R.
CS Vilnius Gediminas Technical University, Department of Chemistry and Bioengineering, Sauletekio 11, Vilnius, LT-10223, Lithuania. irmaorentaite@gmail.com
SO Biochimica et Biophysica Acta - Bioenergetics, (October 2012) Vol. 1817, Supp. SUPPL. 1, pp. S158-S159.
Editor: Friedrich, T.
Editor: Einsle, O.
Editor: Graber, P.
Meeting Info: 17th European Bioenergetics Conference, EBEC 2012. Freiburg, Germany. 15 Sep 2012-20 Sep 2012
ISSN: 0005-2728
PB Elsevier.
PUI S 0005-2728(12)00601-9
DT Journal; Conference; (Conference Abstract)
PS CONF
LA English
SL English
ED Entered STN: Sep 2012
Last Updated on STN: Sep 2012

AB Certain Saccharomyces cerevisiae strains produce killer toxins which kill sensitive cells. This phenomenon is important for strains to survive in natural and industrial populations. Toxins are able to kill the nonkiller and different type killer yeast belonging to the same or other genera while the producing cells remain immune to the action of its own or relative toxins. Depending on the differences of toxins' molecular features, killer effects and encoding

genetic determinants there are three types of killer systems: K1, K2, and K28. Among those, the mechanism of action of K2 killer is the least understood. In order to describe the mechanism of K2 action it is necessary to analyse not only the intraspecial activity but also its effect on other microorganisms. It is known that some yeast killer strains have antibacterial activity but the performed microbiological tests are not sensitive enough to determine it. Instrumental studies carried out with susceptible yeasts and bacteria are more sensitive. The objective of our work was the evaluation of K2 killer toxins' impact on different microorganisms applying microbiological methods and performing bioluminescence and electrochemical analysis to characterise the energetical state of cells. It was shown that during the action K2 toxin decreases the level of ATP and the gradients of small molecules. Our study demonstrated that measurements of bioluminescence and electrochemical analysis are very useful methods for determination of the sensitivity of cells to toxin and the analysis of killing mechanisms.

L7 ANSWER 11 OF 184 MEDLINE ® on STN DUPLICATE 9
AN 2012318987 MEDLINE Full-text
DN PubMed ID: 22423260
TI Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders.
AU Kelesidis Theodoros; Pothoulakis Charalabos
CS Department of Medicine, Division of Infectious Diseases, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA.
SO Therapeutic advances in gastroenterology, (2012 Mar) Vol. 5, No. 2, pp. 111-25.
Journal code: 101478893. E-ISSN: 1756-2848. L-ISSN: 1756-283X.
Report No.: NLM-PMC3296087.
DOI 10.1177/1756283X11428502
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS NONMEDLINE; PUBMED-NOT-MEDLINE
EM 201208
ED Entered STN: 17 Mar 2012
Last Updated on STN: 24 Aug 2012
Entered Medline: 23 Aug 2012
OSC.G 1 There are 1 MEDLINE records that cite this record
AB Several clinical trials and experimental studies strongly suggest a place for Saccharomyces boulardii as a biotherapeutic agent for the prevention and treatment of several gastrointestinal diseases. S. boulardii mediates responses resembling the protective effects of the normal healthy gut flora. The multiple mechanisms of action of S. boulardii and its properties may explain its efficacy and beneficial effects in acute and chronic gastrointestinal diseases that have been confirmed by clinical trials. Caution should be taken in patients with risk factors for adverse events. This review discusses the evidence for efficacy and safety of S. boulardii as a probiotic for the prevention and therapy of gastrointestinal disorders in humans.

L7 ANSWER 12 OF 184 MEDLINE ® on STN DUPLICATE 10
AN 2012769359 MEDLINE Full-text
DN PubMed ID: 22759515
TI Expression of recombinant EARLI1, a hybrid proline-rich protein of Arabidopsis, in Escherichia coli and its inhibition effect to the growth of fungal pathogens and Saccharomyces cerevisiae.
AU Li Lan; Zhang Chen; Xu Dan; Schlappi Michael; Xu Zi-Qin
CS Key Laboratory of Resource Biology and Biotechnology in Western China (Ministry of Education), Provincial Key Laboratory of Biotechnology, Institute of Life Sciences, Northwest University, Xi'an, Shaanxi 710069, People's Republic of China.
SO Gene, (2012 Sep 10) Vol. 506, No. 1, pp. 50-61. Electronic Publication Date: 1 Jul 2012
Journal code: 7706761. E-ISSN: 1879-0038. L-ISSN: 0378-1119.
DOI 10.1016/j.gene.2012.06.070
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201210
ED Entered STN: 5 Jul 2012
Last Updated on STN: 26 Oct 2012
Entered Medline: 25 Oct 2012
AB EARLI1 is an Arabidopsis gene with pleiotropic effects previously shown to have auxiliary functions in protecting plants against freezing-induced cellular damage and promoting germinability under low-temperature and salinity stresses. Here we determined whether recombinant EARLI1 protein has anti-fungal activity. Recombinant EARLI1 protein lacking its signal peptide was produced in Escherichia coli BL21(DE3) using isopropyl β-d-1-thiogalactopyranoside (IPTG) induction and the prokaryotic expression vector pET28a. Expression of EARLI1 was analyzed by Western blotting and the protein was purified using affinity chromatography. Recombinant EARLI1 protein was applied to fungal cultures of Saccharomyces cerevisiae, Botrytis cinerea and Fusarium oxysporum, and membrane permeability was determined using SYTOX green. Full-length EARLI1 was expressed in S. cerevisiae from the GAL1 promoter using 2% galactose and yeast cell viability was compared to control cells. Our results indicated that application of recombinant EARLI1 protein to B. cinerea and F. oxysporum could inhibit the growth of the necrotrophic fungi. Besides, addition of the recombinant protein to liquid cultures of S. cerevisiae significantly suppressed yeast growth and cell viability by increasing membrane permeability, and in vivo expression of the secreted form of EARLI1 in S. cerevisiae also had a remarkable inhibition effect on the growth of yeast cells.

L7 ANSWER 13 OF 184 MEDLINE ® on STN DUPLICATE 11
AN 2012133522 MEDLINE Full-text
DN PubMed ID: 22125264
TI Possible involvement of GDI1 protein, a GDP dissociation inhibitor related to vesicle transport, in an amelioration of zinc toxicity in Saccharomyces cerevisiae.
AU Ezaki Bunichi; Nakakihara Eri
CS Institute of Plant Science and Resources, Okayama University, Japan. bezaki@rib.okayama-u.ac.jp
SO Yeast (Chichester, England), (2012 Jan) Vol. 29, No. 1, pp. 17-24.
Electronic Publication Date: 28 Nov 2011

Journal code: 8607637. E-ISSN: 1097-0061. L-ISSN: 0749-503X.
DOI 10.1002/yea.1913
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201204
ED Entered STN: 12 Jan 2012
Last Updated on STN: 24 Apr 2012
Entered Medline: 23 Apr 2012
AB The GDI1 protein related vesicle transport system was studied to investigate the possibility that an exclusion of toxic zinc (Zn) from the cytoplasm ameliorates Zn toxicity in Saccharomyces cerevisiae (yeast). A temperature-sensitive gdi1 mutant (originally called sec19), in which the GDP dissociation inhibitor becomes inactive at the non-permissive temperature (37 °C), was more sensitive to Zn than its parental GDI1 strain at 32 °C (a moderately non-permissive temperature). The relative efflux of cytoplasmic Zn in the gdi1 mutant was lower than that in the control strain. Treatment with a vesicle transport-specific inhibitor, Brefeldin A, caused an increase of Zn sensitivity and a decrease of Zn efflux in these strains. It is therefore suggested that the GDI1-related vesicle transport system contributes to Zn tolerance in yeast. Furthermore, changes in the number of Zn-specific fluorescent granules (zincosomes) were observed by zinquin staining in the mutant cells under Zn treatment at 32 °C and 37 °C. We concluded that the GDI1 protein is implicated in control of vesicle numbers. Collectively, the results suggest that the GDI1protein is involved in Zn efflux via small vesicle trafficking and contributes to the control of cytoplasmic Zn content, allowing yeast to survive in the presence of toxic Zn.

L7 ANSWER 14 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2012:706507 BIOSIS Full-text
DN PREV201200706507
TI Cch1p Mediates Ca2+ Influx to Protect Saccharomyces cerevisiae against Eugenol Toxicity.
AU Roberts, Stephen K. [Reprint Author]; McAinsh, Martin; Widdicks, Lisa
CS Univ Lancaster, Fac Hlth and Med, Div Biomed and Life Sci, Lancaster, UK
s.k.roberts@lancaster.ac.uk
SO PLoS One, (SEP 13 2012) Vol. 7, No. 9, pp. Article No.: e43989.
ISSN: 1932-6203. E-ISSN: 1932-6203.
DT Article
LA English
ED Entered STN: 31 Oct 2012
Last Updated on STN: 31 Oct 2012
AB Eugenol has antifungal activity and is recognised as having therapeutic potential. However, little is known of the cellular basis of its antifungal activity and a better understanding of eugenol tolerance should lead to better exploitation of eugenol in antifungal therapies. The model yeast, Saccharomyces cerevisiae, expressing apoaequorin was used to show that eugenol induces cytosolic Ca2+ elevations. We investigated the eugenol Ca2+ signature in further detail and show that exponentially growing cells exhibit Ca2+ elevation resulting exclusively from the influx of Ca2+ across the plasma membrane whereas in stationary growth phase cells Ca2+ influx from intracellular and extracellular sources contribute to the eugenol-induced Ca2+ elevation. Ca2+ channel deletion yeast mutants were used to identify the pathways mediating Ca2+ influx: intracellular Ca2+ release was mediated by the vacuolar Ca2+ channel, Yvc1p, whereas the Ca2+ influx across the plasma membrane could be resolved into Cch1p-dependent and Cch1p-independent pathways. We show that the growth of yeast devoid the plasma membrane Ca2+ channel, Cch1p, was hypersensitive to eugenol and that this correlated with reduced Ca2+ elevations. Taken together, these results indicate that a cch1p-mediated Ca2+ influx is part of an intracellular signal which protects against eugenol toxicity. This study provides fresh insight into the mechanisms employed by fungi to tolerate eugenol toxicity which should lead to better exploitation of eugenol in antifungal therapies.

L7 ANSWER 15 OF 184 MEDLINE ® on STN DUPLICATE 12
AN 2011850206 MEDLINE Full-text
DN PubMed ID: 21806066
TI Enhanced squalene production by wild-type Saccharomyces cerevisiae strains using safe chemical means.
AU Naziri Eleni; Mantzouridou Fani; Tsimidou Maria Z
CS Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece.
SO Journal of agricultural and food chemistry, (2011 Sep 28) Vol. 59, No. 18, pp. 9980-9. Electronic Publication Date: 23 Aug 2011
Journal code: 0374755. E-ISSN: 1520-5118. L-ISSN: 0021-8561.
DOI 10.1021/jf201328a
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201201
ED Entered STN: 22 Sep 2011
Last Updated on STN: 14 Jan 2012
Entered Medline: 13 Jan 2012
AB Interest is increasing in establishing renewable sources for squalene, a functional lipid, as the conventional ones are limited. In the present study, squalene production was achieved in a wild-type laboratory Saccharomyces cerevisiae strain by two safe chemical means using terbinafine (0.05-0.55 mM) and methyl jasmonate (MJ) (0-1.00 mM). Bioprocess kinetics optimized by response surface methodology and monitored by high-performance liquid chromatography revealed a clear dependence of growth and squalene content (SQC) and yield (SQY) on the above regulators. Maximum SQC (10.02±0.53 mg/g dry biomass) and SQY (20.70±1.00 mg/L) were achieved using 0.442 mM terbinafine plus 0.044 mM MJ after 28 h and 0.300 mM terbinafine after 30 h, respectively. A 10-fold increase in SQY was achieved in comparison to that in the absence of regulator. The ruggedness of optimum conditions for SQY was verified for five industrial strains. The cellular lipid fraction (.apprx.12% of dry biomass) was rich in squalene (12-13%). Results are encouraging toward bioprocess scale up.

L7 ANSWER 16 OF 184 MEDLINE ® on STN DUPLICATE 13
AN 2011862559 MEDLINE Full-text
DN PubMed ID: 21880084
TI Genome-wide association analysis of clinical vs. nonclinical origin

provides insights into Saccharomyces cerevisiae pathogenesis.
AU Muller L A H; Lucas J E; Georgianna D R; McCusker J H
CS Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA.
NC GM070541 (United States NIGMS NIH HHS)
GM081690 (United States NIGMS NIH HHS)
R01 GM070541-04 (United States NIGMS NIH HHS)
R01 GM081690-04 (United States NIGMS NIH HHS)
SO Molecular ecology, (2011 Oct) Vol. 20, No. 19, pp. 4085-97. Electronic Publication Date: 31 Aug 2011
Journal code: 9214478. E-ISSN: 1365-294X. L-ISSN: 0962-1083.
Report No.: NLM-NIHMS313720; NLM-PMC3183415.
DOI 10.1111/j.1365-294X.2011.05225.x
CY England: United Kingdom
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
LA English
FS MEDLINE; Priority Journals
EM 201203
ED Entered STN: 29 Sep 2011
Last Updated on STN: 27 Mar 2012
Entered Medline: 26 Mar 2012
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 53 There are 53 cited references available in MEDLINE for this document.
AB Because domesticated Saccharomyces cerevisiae strains have been used to produce fermented food and beverages for centuries without apparent health implications, S. cerevisiae has always been considered a Generally Recognized As Safe (GRAS) microorganism. However, the number of reported mucosal and systemic S. cerevisiae infections in the human population has increased and fatal infections have occurred even in relatively healthy individuals. In order to gain insight into the pathogenesis of S. cerevisiae and improve our understanding of the emergence of fungal pathogens, we performed a population-based genome-wide environmental association analysis of clinical vs. nonclinical origin in S. cerevisiae. Using tiling array-based, high-density genotypes of 44 clinical and 44 nonclinical S. cerevisiae strains from diverse geographical origins and source substrates, we identified several genetic loci associated with clinical background in S. cerevisiae. Associated polymorphisms within the coding sequences of VRP1, KIC1, SBE22 and PDR5, and the 5' upstream region of YGR146C indicate the importance of pseudohyphal formation, robust cell wall maintenance and cellular detoxification for S. cerevisiae pathogenesis, and constitute good candidates for follow-up verification of virulence and virulence-related factors underlying the pathogenicity of S. cerevisiae.

L7 ANSWER 17 OF 184 MEDLINE ® on STN DUPLICATE 14
AN 2012046037 MEDLINE Full-text
DN PubMed ID: 22033143
TI The Ashbya gossypii EF-1α promoter of the ubiquitously used MX cassettes is toxic to Saccharomyces cerevisiae.
AU Babazadeh Roja; Jafari Soode Moghadas; Zackrisson Martin; Blomberg Anders; Hohmann Stefan; Warringer Jonas; Krantz Marcus
CS Department of Cell and Molecular Biology, University of Gothenburg, Gothenburg, Sweden.
SO FEBS letters, (2011 Dec 15) Vol. 585, No. 24, pp. 3907-13. Electronic Publication Date: 22 Oct 2011
Journal code: 0155157. E-ISSN: 1873-3468. L-ISSN: 0014-5793.
DOI 10.1016/j.febslet.2011.10.029
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201201
ED Entered STN: 6 Dec 2011
Last Updated on STN: 31 Jan 2012
Entered Medline: 26 Jan 2012
AB Protein overexpression based on introduction of multiple gene copies is well established. To improve purification or quantification, proteins are typically fused to peptide tags. In Saccharomyces cerevisiae, this has been hampered by multicopy toxicity of the TAP and GFP cassettes used in the global strain collections. Here, we show that this effect is due to the EF-1α promoter in the HIS3MX marker cassette rather than the tags per se. This promoter is frequently used in heterologous marker cassettes, including HIS3MX, KanMX, NatMX, PatMX and HphMX. Toxicity could be eliminated by promoter replacement or exclusion of the marker cassette. To our knowledge, this is the first report of toxicity caused by introduction of a heterologous promoter alone.

L7 ANSWER 18 OF 184 MEDLINE ® on STN DUPLICATE 15
AN 2012128771 MEDLINE Full-text
DN PubMed ID: 22228993
TI Grafting of a novel gold(III) complex on nanoporous MCM-41 and evaluation of its toxicity in Saccharomyces cerevisiae.
AU Fazaeli Yousef; Amini Mostafa M; Ashourion Hamed; Heydari Homayoun; Majdabadi Abbas; Jalilian Amir Reza; Abolmaali Shamsozoha
CS Department of Chemistry, Faculty of Sciences, Shahid Beheshti University, Evin, Tehran, Iran.
SO International journal of nanomedicine, (2011) Vol. 6, pp. 3251-7.
Electronic Publication Date: 12 Dec 2011
Journal code: 101263847. E-ISSN: 1178-2013. L-ISSN: 1176-9114.
Report No.: NLM-PMC3252673.
DOI 10.2147/IJN.S25449
CY New Zealand
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201205
ED Entered STN: 10 Jan 2012

Last Updated on STN: 2 May 2012
Entered Medline: 1 May 2012

AB The goal of this research was to investigate the potential of newly synthesized gold complex trichloro(2,4,6-trimethylpyridine)Au(III) as an anticancer agent. The gold(III) complex was synthesized and grafted on nanoporous silica, MCM-41, to produce AuCl(3)@PF-MCM- 41 (AuCl(3) grafted on pyridine-functionalized MCM-41). The toxicity of trichloro(2,4,6- trimethylpyridine)Au(III) and AuCl(3)@PF-MCM-41 in Saccharomyces cerevisiae (as a model system) was studied. The gold(III) complex showed a mid cytotoxic effect on yeast viability. Using the drug delivery system, nanoporous MCM-41, the gold(III) complex became a strong inhibitor for growth of yeast cells at a very low concentration. Furthermore, the animal tests revealed a high uptake of AuCl(3)@PF-MCM-41 in tumor cells. The stability of the compound was confirmed in human serum.

L7 ANSWER 19 OF 184 MEDLINE ® on STN DUPLICATE 16
AN 2011217132 MEDLINE Full-text
DN PubMed ID: 21239561
TI A new wine Saccharomyces cerevisiae killer toxin (Klus), encoded by a double-stranded rna virus, with broad antifungal activity is evolutionarily related to a chromosomal host gene.
AU Rodriguez-Cousino Nieves; Maqueda Matilde; Ambrona Jesus; Zamora Emiliano; Esteban Rosa; Ramirez Manuel
CS Departamento de Microbiologia (Antiguo Rectorado), Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain.
SO Applied and environmental microbiology, (2011 Mar) Vol. 77, No. 5, pp. 1822-32. Electronic Publication Date: 14 Jan 2011
Journal code: 7605801. E-ISSN: 1098-5336. L-ISSN: 0099-2240.
Report No.: NLM-PMC3067279.
DOI 10.1128/AEM.02501-10
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
OS GENBANK-GU723494
EM 201105
ED Entered STN: 26 Feb 2011
Last Updated on STN: 24 May 2011
Entered Medline: 23 May 2011
OSC.G 3 There are 3 MEDLINE records that cite this record
REM.CNT 41 There are 41 cited references available in MEDLINE for this document.

AB Wine Saccharomyces cerevisiae strains producing a new killer toxin (Klus) were isolated. They killed all the previously known S. cerevisiae killer strains, in addition to other yeast species, including Kluyveromyces lactis and Candida albicans. The Klus phenotype is conferred by a medium-size double-stranded RNA (dsRNA) virus, Saccharomyces cerevisiae virus Mlus (ScV-Mlus), whose genome size ranged from 2.1 to 2.3 kb. ScV-Mlus depends on ScV-L-A for stable maintenance and replication. We cloned and sequenced Mlus. Its genome structure is similar to that of M1, M2, or M28 dsRNA, with a 5'-terminal coding region followed by two internal A-rich sequences and a 3'-terminal region without coding capacity. Mlus positive strands carry cis-acting signals at their 5' and 3' termini for transcription and replication similar to those of killer viruses. The open reading frame (ORF) at the 5' portion codes for a putative preprotoxin with an N-terminal secretion signal, potential Kex2p/Kex1p processing sites, and N-glycosylation sites. No sequence homology was found either between the Mlus dsRNA and M1, M2, or M28 dsRNA or between Klus and the K1, K2, or K28 toxin. The Klus amino acid sequence, however, showed a significant degree of conservation with that of the product of the host chromosomally encoded ORF YFR020W of unknown function, thus suggesting an evolutionary relationship.

L7 ANSWER 20 OF 184 MEDLINE ® on STN DUPLICATE 17
AN 2012015715 MEDLINE Full-text
DN PubMed ID: 21890004
TI Kinetic mechanism of an aldehyde reductase of Saccharomyces cerevisiae that relieves toxicity of furfural and 5-hydroxymethylfurfural.
AU Jordan Douglas B; Braker Jay D; Bowman Michael J; Vermillion Karl E; Moon Jaewoong; Liu Z Lewis
CS US Department of Agriculture, National Center of Agricultural Utilization Research, Peoria, IL, USA. douglas.jordan@ars.usda.gov
SO Biochimica et biophysica acta, (2011 Dec) Vol. 1814, No. 12, pp. 1686-94. Electronic Publication Date: 26 Aug 2011
Journal code: 0217513. ISSN: 0006-3002. L-ISSN: 0006-3002.
DOI 10.1016/j.bbapap.2011.08.011
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 201212
ED Entered STN: 24 Nov 2011
Last Updated on STN: 10 Nov 2012
Entered Medline: 13 Dec 2012
OSC.G 1 There are 1 MEDLINE records that cite this record

AB An effective means of relieving the toxicity of furan aldehydes, furfural (FFA) and 5-hydroxymethylfurfural (HMF), on fermenting organisms is essential for achieving efficient fermentation of lignocellulosic biomass to ethanol and other products. Ari1p, an aldehyde reductase from Saccharomyces cerevisiae, has been shown to mitigate the toxicity of FFA and HMF by catalyzing the NADPH-dependent conversion to corresponding alcohols, furfuryl alcohol (FFOH) and 5-hydroxymethylfurfuryl alcohol (HMFOH). At pH 7.0 and 25°C, purified Ari1p catalyzes the NADPH-dependent reduction of substrates with the following values (k(cat) (s(-1)), k(cat)/K(m) (s(-1)mM(-1)), K(m) (mM)): FFA (23.3, 1.82, 12.8), HMF (4.08, 0.173, 23.6), and dl-glyceraldehyde (2.40, 0.0650, 37.0). When acting on HMF and dl-glyceraldehyde, the enzyme operates through an equilibrium ordered kinetic mechanism. In the physiological direction of the reaction, NADPH binds first and NADP(+) dissociates from the enzyme last, demonstrated by k(cat) of HMF and dl-glyceraldehyde that are independent of [NADPH] and (K(ia)(NADPH)/k(cat)) that extrapolate to zero at saturating HMF or dl-glyceraldehyde concentration. Microscopic kinetic parameters were determined for the HMF reaction (HMF+NADPH↔HMFOH+NADP(+)), by applying steady-state, presteady-state, kinetic isotope effects, and dynamic modeling methods. Release of products, HMFOH and NADP(+), is 84% rate limiting to k(cat) in the forward direction. Equilibrium constants, [NADP(+)][FFOH]/[NADPH][FFA][H(+)]=5600×10(7)M(-1) and [NADP(+)][HMFOH]/[NADPH][HMF][H(+)]=4200×10(7)M(-1), favor the physiological direction mirrored by the slowness of hydride transfer in the non-physiological direction, NADP(+)-dependent oxidation of alcohols (k(cat) (s(-1)), k(cat)/K(m) (s(-1)mM(-1)), K(m) (mM)): FFOH (0.221, 0.00158, 140) and HMFOH (0.0105, 0.000104, 101). Published by Elsevier B.V.

L7 ANSWER 21 OF 184 MEDLINE ® on STN DUPLICATE 18
AN 2012038604 MEDLINE Full-text
DN PubMed ID: 22129753
TI A screening for essential cell growth-related genes involved in arsenite toxicity in Saccharomyces cerevisiae.
AU Takahashi Tsutomu; Satake Shohei; Hirose Ken-ichiro; Hwang Gi-Wook; Naganuma Akira
CS Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences,Tohoku University, Sendai, Miyagi, Japan.
SO The Journal of toxicological sciences, (2011) Vol. 36, No. 6, pp. 859-61. Journal code: 7805798. E-ISSN: 1880-3989. L-ISSN: 0388-1350.
CY Japan
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 201203
ED Entered STN: 2 Dec 2011
Last Updated on STN: 28 Mar 2012
Entered Medline: 27 Mar 2012
AB Genes that are essential for growth in yeast were screened to identify those involved in arsenite sensitivity. We found that the knockdown of YPT1, ERG8, or RKI1 enhanced arsenite sensitivity in yeast.

L7 ANSWER 22 OF 184 MEDLINE ® on STN DUPLICATE 19
AN 2011671168 MEDLINE Full-text
DN PubMed ID: 21457450
TI Cell density-dependent linoleic acid toxicity to Saccharomyces cerevisiae.
AU Ferreira Tulio Cesar; de Moraes Lidia Maria Pepe; Campos Elida Geralda
CS Departamento de Biologia Celular, Universidade de Brasilia, Distrito Federal, Brazil. tulcesar@gmail.com
SO FEMS yeast research, (2011 Aug) Vol. 11, No. 5, pp. 408-17. Electronic Publication Date: 26 Apr 2011
Journal code: 101085384. E-ISSN: 1567-1364. L-ISSN: 1567-1356.
DOI 10.1111/j.1567-1364.2011.00729.x
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201203
ED Entered STN: 13 Jul 2011
Last Updated on STN: 17 Mar 2012
Entered Medline: 16 Mar 2012
AB Since the discovery of the apoptotic pathway in Saccharomyces cerevisiae, several compounds have been shown to cause apoptosis in this organism. While the toxicity of polyunsaturated fatty acids (PUFA) peroxides towards S. cerevisiae has been known for a long time, studies on the effect of nonoxidized PUFA are scarce. The present study deals specifically with linoleic acid (LA) in its nonoxidized form and investigates its toxicity to yeast. Saccharomyces cerevisiae is unable to synthesize PUFA, but can take up and incorporate them into its membranes. Reports from the literature indicate that LA is not toxic to yeast cells. However, we demonstrated that yeast cell growth decreased in cultures treated with 0.1 mM LA for 4 h, and 3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction (a measure of respiratory activity) decreased by 47%. This toxicity was dependent on the number of cells used in the experiment. We show apoptosis induction by LA concomitant with increases in malondialdehyde, glutathione content, activities of catalase and cytochrome c peroxidase, and decreases in two metabolic enzyme activities. While the main purpose of this study was to show that LA causes cell death in yeast, our results indicate some of the molecular mechanisms of the cell toxicity of PUFA. .COPYRGT. 2011 Federation of European Microbiological Societies. Published by Blackwell Publishing Ltd. All rights reserved.

L7 ANSWER 23 OF 184 MEDLINE ® on STN DUPLICATE 20
AN 2011034621 MEDLINE Full-text
DN PubMed ID: 21081253
TI In vivo virulence of commercial Saccharomyces cerevisiae strains with pathogenicity-associated phenotypical traits.
AU de Llanos R; Llopis S; Molero G; Querol A; Gil C; Fernandez-Espinar M T
CS Departamento de Biotecnologia, Instituto de Agroquimica y Tecnologia de los Alimentos CSIC, PO Box 73, 46100 Burjassot, Valencia, Spain.
SO International journal of food microbiology, (2011 Jan 5) Vol. 144, No. 3, pp. 393-9. Electronic Publication Date: 28 Oct 2010
Journal code: 8412849. E-ISSN: 1879-3460. L-ISSN: 0168-1605.
DOI 10.1016/j.ijfoodmicro.2010.10.025
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201106
ED Entered STN: 18 Dec 2010
Last Updated on STN: 3 Jun 2011
Entered Medline: 2 Jun 2011
OSC.G 2 There are 2 MEDLINE records that cite this record
AB Two commercial Saccharomyces cerevisiae strains, a baker's strain and the bio-therapeutic agent Ultralevure, have been proposed as a possible exogenous source of human colonization (de Llanos et al., 2004, 2006a). Moreover, these strains express phenotypical traits associated to pathogenicity (de Llanos et al., 2006b). Taking into account that both commercial preparations represent an important source of living S. cerevisiae cells we have performed an in vivo study to evaluate whether there is a potential safety risk to humans. Their virulence was compared with that of other commercial strains with less virulent traits, and with clinical isolates, using two murine models (BALB/c and DBA/2N mice). Burden determination in the brain and kidneys showed that the ability to disseminate, colonize and persist was manifested not only by clinical isolates but also by commercial strains. Among these, the baker's strain and Ultralevure were able to cause the death of BALB/c mice at rates similar to those shown by two of the clinical isolates. These results highlight the pathogenic potential of these strains and show that four-week-old BALB/c mice are an appropriate murine model to study the virulence of yeasts with low or moderate pathogenicity. Furthermore, we have shown the positive effect of an immunosuppressive therapy with cyclophosphamide in the virulence of the baker's strains and Ultralevure but not in the rest of the commercial strains under study. The data suggest that although S.

cerevisiae has always been considered a GRAS microorganism, commercial preparations should include only those strains shown to be safe in order to minimize complications in risk groups. Copyright .COPYRGT. 2010 Elsevier B.V. All rights reserved.

L7 ANSWER 24 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 21
AN 2012:356677 BIOSIS Full-text
DN PREV201200356677
TI Grafting of gold complex on nano-porous MCM-41 and evaluation of its toxicity in Saccharomyces cerevisiae as a model system.
AU Fazaeli, Yousef; Amini, Mostafa M.; Ashoorion, Hamed; Jalilian, Amirreza; Majdabadi, Abbas; Rahiminezhad, Ali; Shamsozzoha, Abolmaali
CS youseffazaeli@gmail.com
SO Clinical Biochemistry, (SEP 2011) Vol. 44, No. 13, Suppl. S, pp. S363. Meeting Info.: 12th Iranian Congress of Biochemistry (ICB)/4th International Congress of Biochemistry and Molecular Biology (ICBMB). Mashhad, IRAN. September 06 -09, 2011.
CODEN: CLBIAS. ISSN: 0009-9120. E-ISSN: 1873-2933.
DT Conference; (Meeting)
Conference; (Meeting Poster)
LA English
ED Entered STN: 9 May 2012
Last Updated on STN: 9 May 2012

L7 ANSWER 25 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 22
AN 2011:595974 BIOSIS Full-text
DN PREV201100595974
TI Toxicity of isoproturon on Saccharomyces cerevisiae growing in mineral medium depends on glutathione-mediated antioxidant capacity.
AU Candeias, M. S. G. [Reprint Author]; Alves-Pereira, I. M. S.; Ferreira, R. M. A.
CS Univ Evora, Inst Ciencias Agrarias and Ambientais Mediterran IC, Evora, Portugal
SO Toxicology Letters (Shannon), (AUG 28 2011) Vol. 205, No. Suppl. 1, pp. S229.
Meeting Info.: 47th Congress of the European-Societies-of-Toxicology. Paris, FRANCE. August 28 -31, 2011. European Soc Toxicol.
CODEN: TOLED5. ISSN: 0378-4274.
DT Conference; (Meeting)
Conference; Abstract; (Meeting Abstract)
LA English
ED Entered STN: 14 Sep 2011
Last Updated on STN: 14 Sep 2011

L7 ANSWER 26 OF 184 MEDLINE ® on STN DUPLICATE 23
AN 2011116785 MEDLINE Full-text
DN PubMed ID: 21114626
TI Transcriptomic and phenotypic analysis of the effects of T-2 toxin on Saccharomyces cerevisiae: evidence of mitochondrial involvement.
AU Josse Lyne; Li Xingmin; Coker Raymond D; Gourlay Campbell W; Evans Ivor H
CS School of Biosciences, Ingram Building, University of Kent, Canterbury, Kent, UK.
SO FEMS yeast research, (2011 Feb) Vol. 11, No. 1, pp. 133-50. Electronic Publication Date: 29 Nov 2010
Journal code: 101085384. E-ISSN: 1567-1364. L-ISSN: 1567-1356.
DOI 10.1111/j.1567-1364.2010.00699.x
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 201104
ED Entered STN: 18 Jan 2011
Last Updated on STN: 21 Apr 2011
Entered Medline: 20 Apr 2011

AB At 5 µg mL(-1) , T-2 toxin significantly upregulated the transcription of 281 genes and downregulated 86. Strongly upregulated genes included those involved in redox activity, mitochondrial functions, the response to oxidative stress, and cytoplasmic rRNA transcription and processing. Highly repressed genes have roles in mitochondrial biogenesis, and the expression and stability of cytoplasmic rRNAs. T-2 toxin inhibition of growth was greater in a medium requiring respiration, and was antagonized by antioxidants. T-2 toxin treatment induced reactive oxygen species, caused nucleolytic damage to DNA, probably mitochondrial, and externalization of phosphatidylserine. Deletion mutations causing respiratory deficiency substantially increased toxin tolerance, and deletion of some TOR (target of rapamycin) pathway genes altered T-2 toxin sensitivity. Deletion of FMS1, which plays an indirect role in cytoplasmic protein synthesis, markedly increased toxin tolerance. Overall, the findings suggest that T-2 toxin targets mitochondria, generating oxy-radicals and repressing mitochondrial biogenesis genes, thus inducing oxidative stress and redox enzyme genes, and triggering changes associated with apoptosis. The large transcriptional changes in genes needed for rRNA transcription and expression and the effects of deletion of FMS1 are also consistent with T-2 toxin damage to the cytoplasmic translational mechanism, although it is unclear how this relates to the mitochondrial effects. .COPYRGT. 2010 Federation of European Microbiological Societies. Published by Blackwell Publishing Ltd. All rights reserved.

L7 ANSWER 27 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 24
AN 2011:301315 BIOSIS Full-text
DN PREV201100301315
TI Role of the Ubiquitin-proteasome System in Methylmercury Toxicity in Saccharomyces cerevisiae.
AU Hwang, Gi-Wook [Reprint Author]
CS Tohoku Univ, Lab Mol and Biochem Toxicol, Grad Pharmaceut Sci, Aoba Ku, 6-3 Aza Aoba, Sendai, Miyagi 9808578, Japan
gwhwang@mail.pharm.tohoku.ac.jp
SO Journal of Health Science, (APR 2011) Vol. 57, No. 2, pp. 129-133.
ISSN: 1344-9702. E-ISSN: 1347-5207.

DT Article
General Review; (Literature Review)
LA English
ED Entered STN: 11 May 2011
Last Updated on STN: 13 Jun 2012
AB Methylmercury (MeHg) is an important environmental pollutant that causes severe disorders of the central nervous system, but the mechanism underlying its toxicity and the corresponding biological defense mechanisms remain largely unknown. Saccharomyces cerevisiae (S. cererisiae) yeast cells were used to elucidate the defense mechanisms against MeHg toxicity and to search for novel genes involved in MeHg resistance. S. cerevisiae is a eukaryotic organism that possesses many gene products that are functionally similar to those of mammals such as humans. We have previously reported that Cdc34 and Rad23 confer MeHg resistance to yeast cells. Interestingly, the both proteins are related to ubiquitin-proteasome system (UP system) that is involved in the intracellular degradation of proteins. In our detailed experiments, we found that the UP system might play an important role in lending protection against MeHg toxicity. This review summarizes the results of our studies on the role of the UP system as a defense mechanism against MeHg toxicity in yeast cells.

L7 ANSWER 28 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 0050616760 EMBASE Full-text
TI Sulfate assimilation mediates tellurite reduction and toxicity in Saccharomyces cerevisiae.
AU Ottosson, Lars-Goran (correspondence); Logg, Katarina; Ibstedt, Sebastian; Sunnerhagen, Per; Blomberg, Anders; Warringer, Jonas
CS Department of Cell and Molecular Biology, U. of Gothenburg, Sweden.
AU Logg, Katarina; Kll, Mikael
CS Department of Applied Physics, Chalmers U. of Technology, Gothenburg, Sweden.
AU Warringer, Jonas
CS Centre for Integrative Genetics, Norwegian U. of Life Sciences As, Norway.
SO Yeast, (July 2011) Vol. 28, Supp. SUPPL. 1, pp. S78. Abstract Number: 05-27.
Meeting Info: 25th International Conference on Yeast Genetics and Molecular Biology. Olsztyn, Poland. 11 Jul 2011-16 Jul 2011
ISSN: 0749-503X
PB John Wiley and Sons Ltd.
DT Journal; Conference; (Conference Abstract)
FS CONF
LA English
SL English
ED Entered STN: Sep 2012
Last Updated on STN: Sep 2012
AB Despite a century of research and increasing environmental and human health concerns, the mechanistic basis for the toxicity and intracellular accumulation of derivates of the metalloid tellurium, in particular the oxyanion tellurite, remains unsolved. Here, we provide an unbiased view of mechanisms of tellurium metabolism in the yeast Saccharomyces cerevisiae by measuring deviations in tellurium tolerance and accumulation of a complete collection of gene knockouts. Reduction of tellurite and concomitant intracellular accumulation as metallic tellurium strongly correlated with loss of cellular fitness, demonstrating that tellurite reduction and toxicity are causally linked. The sulfate assimilation pathway upstream of Met17 and in particular the sulfite reductase and its cofactor siroheme were shown to be central to tellurite toxicity and its reduction to elemental tellurium. Gene knockouts with a deviation in tellurite tolerance also showed a similar deviation in the tolerance to both selenite and, interestingly, selenomethionine. This suggests that the bioassimilation of selenium and tellurium into seleno- and telluromethionine via the sulfate assimilation pathway, with potential further incorporation into proteins, is an important cause of tellurite and selenite toxicity in yeast. The here reported results represent a robust base from which to attack the mechanistic details of this molecular enigma.

L7 ANSWER 29 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 0050616716 EMBASE Full-text
TI A genetic screeening for suppressors of the human caspase-10 toxicity in Saccharomyces cerevisiae uncovers novel functions of FAR11.
AU Jimenez, Alberto (correspondence); Lisa-Santamaria, Patricia; Cifuentes-Esquivel, Alejandra; Revuelta, Jose Luis
CS University of Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain.
SO Yeast, (July 2011) Vol. 28, Supp. SUPPL. 1, pp. S54. Abstract Number: 03-1.
Meeting Info: 25th International Conference on Yeast Genetics and Molecular Biology. Olsztyn, Poland. 11 Jul 2011-16 Jul 2011
ISSN: 0749-503X
PB John Wiley and Sons Ltd.
DT Journal; Conference; (Conference Abstract)
FS CONF
LA English
SL English
ED Entered STN: Sep 2012
Last Updated on STN: Sep 2012
AB Humanized yeast have been used extensively in functional studies of mammalian proteins and have allowed unraveling novel functions and regulation mechanisms of human genes with or without fungal counterparts. Recent studies have revealed that apoptosis and autophagy share common pathways and effectors and that certain stimuli may trigger mixed phenotypes of apoptosis and autophagy at the cellular level. The heterologous expression of the human caspase-10 in S. cerevisiae induces a type of cell death associated with some apoptotic and autophagic hallmarks. We carried out a genetic screen for suppressors of the human caspase-10 toxicity using an haploid YKO collection. Our results indicate that both the pheromone signaling and autophagy pathways are essential for the caspase-10 induced lethality in S. cerevisiae. Here we focused on the involvement of the yeast FAR11 gene, which has two human orthologs of unknown function, in the caspase-10 citotoxicity. Far11 localizes in the membrane of late Golgi vesicles and we have found that Far11 contributes to the induction of cell death possibly by modulating the initiation of autophagy. Additionally, Far11 participates in the cellular response against DNA damage through the regulation of the intra-S checkpoint.

L7 ANSWER 30 OF 184 MEDLINE ® on STN DUPLICATE 25
AN 2011062925 MEDLINE Full-text
DN PubMed ID: 20803478

TI Genome-wide screen of Saccharomyces cerevisiae for killer toxin HM-1 resistance.
AU Miyamoto Masahiko; Furuichi Yasuhiro; Komiyama Tadazumi
CS Department of Biochemistry, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima, Akiha-ku, Niigata 956-8603, Japan. miyamoto@nupals.ac.jp
SO Yeast (Chichester, England), (2011 Jan) Vol. 28, No. 1, pp. 27-41. Electronic Publication Date: 27 Aug 2010
Journal code: 8607637. E-ISSN: 1097-0061. L-ISSN: 0749-503X.
DOI 10.1002/yea.1818
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 201103
ED Entered STN: 25 Dec 2010
Last Updated on STN: 1 Apr 2011
Entered Medline: 31 Mar 2011
AB We screened a set of Saccharomyces cerevisiae deletion mutants for resistance to killer toxin HM-1, which kills susceptible yeasts through inhibiting 1,3-beta-glucan synthase. By using HM-1 plate assay, we found that eight gene-deletion mutants had higher HM-1-resistance compared with the wild-type. Among these eight genes, five--ALG3, CAX4, MNS1, OST6 and YBL083C--were associated with N-glycan formation and maturation. The ALG3 gene has been shown before to be highly resistant to HM-1. The YBL083C gene may be a dubious open reading frame that overlaps partially the ALG3 gene. The deletion mutant of the MNS1 gene that encodes 1,2-alpha-mannosidase showed with a 13-fold higher HM-1 resistance compared with the wild-type. By HM-1 binding assay, the yeast plasma membrane fraction of alg3 and mns1 cells had less binding ability compared with wild-type cells. These results indicate that the presence of the terminal 1,3-alpha-linked mannose residue of the B-chain of the N-glycan structure is essential for interaction with HM-1. A deletion mutant of aquaglyceroporin Fps1p also showed increased HM-1 resistance. A deletion mutant of osmoregulatory mitogen-activated protein kinase Hog1p was more sensitive to HM-1, suggesting that high-osmolarity glycerol pathways plays an important role in the compensatory response to HM-1 action. Copyright .COPYRGT. 2010 John Wiley & Sons, Ltd.

L7 ANSWER 31 OF 184 MEDLINE ® on STN DUPLICATE 26
AN 2011016827 MEDLINE Full-text
DN PubMed ID: 21124936
TI The cytosolic tail of the Golgi apyrase Ynd1 mediates E4orf4-induced toxicity in Saccharomyces cerevisiae.
AU Mittelman Karin; Ziv Keren; Maoz Tsofnat; Kleinberger Tamar
CS Department of Molecular Microbiology, Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel.
SO PloS one, (2010) Vol. 5, No. 11, pp. e15539. Electronic Publication Date: 22 Nov 2010
Journal code: 101285081. E-ISSN: 1932-6203. L-ISSN: 1932-6203.
Report No.: NLM-PMC2989921.
DOI 10.1371/journal.pone.0015539
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201104
ED Entered STN: 7 Dec 2010
Last Updated on STN: 3 May 2011
Entered Medline: 27 Apr 2011
AB The adenovirus E4 open reading frame 4 (E4orf4) protein contributes to regulation of the progression of virus infection. When expressed individually, E4orf4 was shown to induce non-classical transformed cell-specific apoptosis in mammalian cells. At least some of the mechanisms underlying E4orf4-induced toxicity are conserved from yeast to mammals, including the requirement for an interaction of E4orf4 with protein phosphatase 2A (PP2A). A genetic screen in yeast revealed that the Golgi apyrase Ynd1 associates with E4orf4 and contributes to E4orf4-induced toxicity, independently of Ynd1 apyrase activity. Ynd1 and PP2A were shown to contribute additively to E4orf4-induced toxicity in yeast, and to interact genetically and physically. A mammalian orthologue of Ynd1 was shown to bind E4orf4 in mammalian cells, confirming the evolutionary conservation of this interaction. Here, we use mutation analysis to identify the cytosolic tail of Ynd1 as the protein domain required for mediation of the E4orf4 toxic signal and for the interaction with E4orf4. We also show that E4orf4 associates with cellular membranes in yeast and is localized at their cytoplasmic face. However, E4orf4 is membrane-associated even in the absence of Ynd1, suggesting that additional membrane proteins may mediate E4orf4 localization. Based on our results and on a previous report describing a collection of Ynd1 protein partners, we propose that the Ynd1 cytoplasmic tail acts as a scaffold, interacting with a multi-protein complex, whose targeting by E4orf4 leads to cell death.

L7 ANSWER 32 OF 184 MEDLINE ® on STN DUPLICATE 27
AN 2010864899 MEDLINE Full-text
DN PubMed ID: 20675578
TI Sulfate assimilation mediates tellurite reduction and toxicity in Saccharomyces cerevisiae.
AU Ottosson Lars-Goran; Logg Katarina; Ibstedt Sebastian; Sunnerhagen Per; Kall Mikael; Blomberg Anders; Warringer Jonas
CS Department of Cell and Molecular Biology, University of Gothenburg, Sweden.
SO Eukaryotic cell, (2010 Oct) Vol. 9, No. 10, pp. 1635-47. Electronic Publication Date: 30 Jul 2010
Journal code: 101130731. E-ISSN: 1535-9786. L-ISSN: 1535-9786.
Report No.: NLM-PMC2950436.
DOI 10.1128/EC.00078-10
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201101
ED Entered STN: 5 Oct 2010
Last Updated on STN: 29 Jan 2011
Entered Medline: 28 Jan 2011
REM.CNT 62 There are 62 cited references available in MEDLINE for this

document.

AB Despite a century of research and increasing environmental and human health concerns, the mechanistic basis of the toxicity of derivatives of the metalloid tellurium, Te, in particular the oxyanion tellurite, Te(IV), remains unsolved. Here, we provide an unbiased view of the mechanisms of tellurium metabolism in the yeast Saccharomyces cerevisiae by measuring deviations in Te-related traits of a complete collection of gene knockout mutants. Reduction of Te(IV) and intracellular accumulation as metallic tellurium strongly correlated with loss of cellular fitness, suggesting that Te(IV) reduction and toxicity are causally linked. The sulfate assimilation pathway upstream of Met17, in particular, the sulfite reductase and its cofactor siroheme, was shown to be central to tellurite toxicity and its reduction to elemental tellurium. Gene knockout mutants with altered Te(IV) tolerance also showed a similar deviation in tolerance to both selenite and, interestingly, selenomethionine, suggesting that the toxicity of these agents stems from a common mechanism. We also show that Te(IV) reduction and toxicity in yeast is partially mediated via a mitochondrial respiratory mechanism that does not encompass the generation of substantial oxidative stress. The results reported here represent a robust base from which to attack the mechanistic details of Te(IV) toxicity and reduction in a eukaryotic organism.

L7 ANSWER 33 OF 184 MEDLINE ® on STN DUPLICATE 28
AN 2010970567 MEDLINE Full-text
DN PubMed ID: 20809078
TI Lead toxicity in Saccharomyces cerevisiae.
AU Van der Heggen Maarten; Martins Sara; Flores Gisela; Soares Eduardo V
CS Bioengineering Laboratory, Chemical Engineering Department, Superior Institute of Engineering from Porto Polytechnic Institute, Rua Dr Antonio Bernardino de Almeida, 431, 4200-072 Porto, Portugal.
SO Applied microbiology and biotechnology, (2010 Dec) Vol. 88, No. 6, pp. 1355-61. Electronic Publication Date: 1 Sep 2010
Journal code: 8406612. E-ISSN: 1432-0614. L-ISSN: 0175-7598.
DOI 10.1007/s00253-010-2799-5
CY Germany: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 201102
ED Entered STN: 17 Nov 2010
Last Updated on STN: 26 Feb 2011
Entered Medline: 25 Feb 2011

AB The effect of Pb on Saccharomyces cerevisiae cell structure and function was examined. Membrane integrity was assessed by the release of UV-absorbing compounds and by the intracellular K(+) efflux. No leakage of UV(260)-absorbing compounds or loss of K(+) were observed in Pb (until 1,000 μmol/l) treated cells up to 30 min; these results suggest that plasma membrane seems not to be the immediate and primary target of Pb toxicity. The effect of Pb on yeast metabolism was examined using the fluorescent probe FUN-1 and compared with the ability to reproduce, evaluated by colony-forming units counting. The exposition of yeast cells, during 60 min to 1,000 μmol/l Pb, induces a decrease in the ability to process FUN-1 although the cells retain its proliferation capacity. A more prolonged contact time (120 min) of yeast cells with Pb induces a marked (> 50%) loss of yeast cells metabolic activity and replication competence through a mechanism which most likely requires protein synthesis.

L7 ANSWER 34 OF 184 MEDLINE ® on STN DUPLICATE 29
AN 2010935870 MEDLINE Full-text
DN PubMed ID: 20374295
TI How Saccharomyces cerevisiae copes with toxic metals and metalloids.
AU Wysocki Robert; Tamas Markus J
CS Institute of Genetics and Microbiology, University of Wroclaw, Wroclaw, Poland.
SO FEMS microbiology reviews, (2010 Nov) Vol. 34, No. 6, pp. 925-51.
Journal code: 8902526. E-ISSN: 1574-6976. L-ISSN: 0168-6445.
DOI 10.1111/j.1574-6976.2010.00217.x
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
General Review; (REVIEW)
LA English
FS MEDLINE; Priority Journals
EM 201101
ED Entered STN: 3 Nov 2010
Last Updated on STN: 25 Jan 2011
Entered Medline: 24 Jan 2011
OSC.G 6 There are 6 MEDLINE records that cite this record

AB Toxic metals and metalloids are widespread in nature and can locally reach fairly high concentrations. To ensure cellular protection and survival in such environments, all organisms possess systems to evade toxicity and acquire tolerance. This review provides an overview of the molecular mechanisms that contribute to metal toxicity, detoxification and tolerance acquisition in budding yeast Saccharomyces cerevisiae. We mainly focus on the metals/metalloids arsenic, cadmium, antimony, mercury, chromium and selenium, and emphasize recent findings on sensing and signalling mechanisms and on the regulation of tolerance and detoxification systems that safeguard cellular and genetic integrity.

L7 ANSWER 35 OF 184 MEDLINE ® on STN DUPLICATE 30
AN 2010307091 MEDLINE Full-text
DN PubMed ID: 20305002
TI Homoserine toxicity in Saccharomyces cerevisiae and Candida albicans homoserine kinase (thr1Delta) mutants.
AU Kingsbury Joanne M; McCusker John H
CS Department of Molecular Genetics and Microbiology, Box 3020, Duke University Medical Center, Durham, NC 27710, USA.
NC R21 AI070247 (United States NIAID NIH HHS)
R21 AI070247-02 (United States NIAID NIH HHS)
SO Eukaryotic cell, (2010 May) Vol. 9, No. 5, pp. 717-28. Electronic Publication Date: 19 Mar 2010
Journal code: 101130731. E-ISSN: 1535-9786. L-ISSN: 1535-9786.
Report No.: NLM-PMC2863960.
DOI 10.1128/EC.00044-10
CY United States
DT Journal; Article; (JOURNAL ARTICLE)

(RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
LA English
FS MEDLINE; Priority Journals
EM 201007
ED Entered STN: 5 May 2010
Last Updated on STN: 31 Jul 2010
Entered Medline: 30 Jul 2010
OSC.G 1 There are 1 MEDLINE records that cite this record
AB In addition to threonine auxotrophy, mutation of the Saccharomyces cerevisiae threonine biosynthetic genes THR1 (encoding homoserine kinase) and THR4 (encoding threonine synthase) results in a plethora of other phenotypes. We investigated the basis for these other phenotypes and found that they are dependent on the toxic biosynthetic intermediate homoserine. Moreover, homoserine is also toxic for Candida albicans thr1Delta mutants. Since increasing levels of threonine, but not other amino acids, overcome the homoserine toxicity of thr1Delta mutants, homoserine may act as a toxic threonine analog. Homoserine-mediated lethality of thr1Delta mutants is blocked by cycloheximide, consistent with a role for protein synthesis in this lethality. We identified various proteasome and ubiquitin pathway components that either when mutated or present in high copy numbers suppressed the thr1Delta mutant homoserine toxicity. Since the doa4Delta and proteasome mutants identified have reduced ubiquitin- and/or proteasome-mediated proteolysis, the degradation of a particular protein or subset of proteins likely contributes to homoserine toxicity.

L7 ANSWER 36 OF 184 MEDLINE ® on STN DUPLICATE 31
AN 2010120285 MEDLINE Full-text
DN PubMed ID: 19955329
TI Investigations on the role of base excision repair and non-homologous end-joining pathways in sodium selenite-induced toxicity and mutagenicity in Saccharomyces cerevisiae.
AU Manikova Dominika; Vlasakova Danusa; Loduhova Jana; Letavayova Lucia; Vigasova Dana; Krascsenitsova Eva; Vlckova Viera; Brozmanova Jela; Chovanec Miroslav
CS Laboratory of Molecular Genetics, Cancer Research Institute, Vlarska 7, 833 91 Bratislava, Slovak Republic.
SO Mutagenesis, (2010 Mar) Vol. 25, No. 2, pp. 155-62. Electronic Publication Date: 2 Dec 2009
Journal code: 8707812. E-ISSN: 1464-3804. L-ISSN: 0267-8357.
DOI 10.1093/mutage/gep056
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201005
ED Entered STN: 24 Feb 2010
Last Updated on STN: 21 May 2010
Entered Medline: 20 May 2010
OSC.G 3 There are 3 MEDLINE records that cite this record
AB Selenium (Se) belongs to nutrients that are essential for human health. Biological activity of this compound, however, mainly depends on its dose, with a potential of Se to induce detrimental effects at high doses. Although mechanisms lying behind detrimental effects of Se are poorly understood yet, they involve DNA damage induction. Consequently, DNA damage response and repair pathways may play a crucial role in cellular response to Se. Using Saccharomyces cerevisiae we showed that sodium selenite (SeL), an inorganic form of Se, can be toxic and mutagenic in this organism due to its ability to induce DNA double-strand breaks (DSBs). Moreover, we reported that a spectrum of mutations induced by this compound in the stationary phase of growth is mainly represented by 1-4 bp deletions. Consequently, we proposed that SeL acts as an oxidizing agent in yeast producing oxidative damage to DNA. As short deletions could be anticipated to arise as a result of action of non-homologous end-joining (NHEJ) and oxidative damage to DNA is primarily coped with base excision repair (BER), a contribution of these two pathways towards survival, DSB induction, mutation frequency and types of mutations following SeL exposure was examined in present study. First, we show that while NHEJ plays no role in repairing toxic DNA lesions induced by SeL, cells with impairment in BER are sensitized towards this compound. Of BER activities examined, those responsible for processing of 3'-blocking DNA termini seem to be the most crucial for manifestation of the toxic effects of SeL in yeast. Second, an impact of NHEJ and BER on DSB induction after SeL exposure turned to be inappreciable, as no increase in DNA double-strand breakage in NHEJ and BER single or NHEJ BER double mutant upon SeL exposure was observed. Finally, we demonstrate that impairment in both these pathways does not importantly change mutation frequency after SeL exposure and that NHEJ is not responsible for generation of short deletions after SeL treatment, as these were comparably induced in the wild-type and NHEJ-defective cells.

L7 ANSWER 37 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 2011115163 EMBASE Full-text
TI Production and effect of killer toxin by Saccharomyces cerevisiae on sensitive yeast and fungal pathogens.
AU Mohamudha Parveen, R. (correspondence); Ayesha Begum, J.
CS Department of Microbiology, Adhiparasakthi College of Arts and Science, G.B.Nagar, Kalavai, Vellore District- 632506, India. mahamudhaparveen@gmail.com
SO International Journal of Pharmaceutical Sciences Review and Research, (July - August 2010) Vol. 3, No. 1, pp. 127-129.
Refs: 11
E-ISSN: 0976-044X
PB Global Research Online, Plot No: 6, R. K. Lake view, Hebbagudi, Anekal Taluk, Bangalore, India.
CY India
DT Journal; Article
FS 004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
037 Drug Literature Index
LA English
SL English
ED Entered STN: 8 Mar 2011
Last Updated on STN: 8 Mar 2011
AB Killer toxins are the proteinaceous toxins produced by some group of yeast designated- Killer yeasts. These toxins have activity against microorganisms other than yeast and the activity is readily detectable only when a suitable sensitive strain is tested. The aim of this study was extract the Killer toxin from yeast cells, to purify by thin layer chromatography and to find out the action of killer toxin on different fungal pathogens.

L7 ANSWER 38 OF 184 MEDLINE ® on STN DUPLICATE 32
AN 2009816924 MEDLINE Full-text
DN PubMed ID: 19779031
TI VopF, a type III effector protein from a non-O1, non-O139 Vibrio cholerae strain, demonstrates toxicity in a Saccharomyces cerevisiae model.
AU Tripathi Ranjana; Singh Naorem Santa; Dureja Chetna; Haldar Swati; Mondal Alok K; Raychaudhuri Saumya
CS Institute of Microbial Technology, Molecular Biology Division, Chandigarh, Council of Scientific and Industrial Research (CSIR), New Delhi, India.
SO Journal of medical microbiology, (2010 Jan) Vol. 59, No. Pt 1, pp. 17-24. Journal code: 0224131. E-ISSN: 1473-5644. L-ISSN: 0022-2615.
DOI 10.1099/jmm.0.012336-0
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE) (RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201001
ED Entered STN: 22 Dec 2009
Last Updated on STN: 6 Jan 2010
Entered Medline: 5 Jan 2010
OSC.G 1 There are 1 MEDLINE records that cite this record
AB VopF, a type III effector protein, has been identified as a contributory factor to the intestinal colonization of type III secretion system-positive, non-O1, non-O139 Vibrio cholerae strains. To gain more insight into the function of VopF, a yeast model was developed. Using this model, it was found that ectopic expression of VopF conferred toxicity in yeast.

L7 ANSWER 39 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2011:37875 BIOSIS Full-text
DN PREV201100037875
TI The Cytosolic Tail of the Golgi Apyrase Ynd1 Mediates E4orf4-Induced Toxicity in Saccharomyces cerevisiae.
AU Mittelman, Karin [Reprint Author]; Ziv, Keren; Maoz, Tsofnat; Kleinberger, Tamar
CS Weizmann Inst Sci, Dept Mol Genet, IL-76100 Rehovot, Israel
tamark@tx.technion.ac.il
SO PLoS One, (NOV 22 2010) Vol. 5, No. 11, pp. Article No.: e15539. ISSN: 1932-6203.
DT Article
LA English
ED Entered STN: 29 Dec 2010
Last Updated on STN: 29 Dec 2010
AB The adenovirus E4 open reading frame 4 (E4orf4) protein contributes to regulation of the progression of virus infection. When expressed individually, E4orf4 was shown to induce non-classical transformed cell-specific apoptosis in mammalian cells. At least some of the mechanisms underlying E4orf4-induced toxicity are conserved from yeast to mammals, including the requirement for an interaction of E4orf4 with protein phosphatase 2A (PP2A). A genetic screen in yeast revealed that the Golgi apyrase Ynd1 associates with E4orf4 and contributes to E4orf4-induced toxicity, independently of Ynd1 apyrase activity. Ynd1 and PP2A were shown to contribute additively to E4orf4-induced toxicity in yeast, and to interact genetically and physically. A mammalian orthologue of Ynd1 was shown to bind E4orf4 in mammalian cells, confirming the evolutionary conservation of this interaction. Here, we use mutation analysis to identify the cytosolic tail of Ynd1 as the protein domain required for mediation of the E4orf4 toxic signal and for the interaction with E4orf4. We also show that E4orf4 associates with cellular membranes in yeast and is localized at their cytoplasmic face. However, E4orf4 is membrane-associated even in the absence of Ynd1, suggesting that additional membrane proteins may mediate E4orf4 localization. Based on our results and on a previous report describing a collection of Ynd1 protein partners, we propose that the Ynd1 cytoplasmic tail acts as a scaffold, interacting with a multi-protein complex, whose targeting by E4orf4 leads to cell death.

L7 ANSWER 40 OF 184 MEDLINE ® on STN DUPLICATE 33
AN 2009538145 MEDLINE Full-text
DN PubMed ID: 19502579
TI A pathogenesis assay using Saccharomyces cerevisiae and Caenorhabditis elegans reveals novel roles for yeast AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis.
AU Jain Charu; Yun Meijiang; Politz Samuel M; Rao Reeta Prusty
CS Department of Biology and Biotechnology, Life Sciences and Bioengineering Center at Gateway Park, Worcester Polytechnic Institute, MA 01605, USA.
SO Eukaryotic cell, (2009 Aug) Vol. 8, No. 8, pp. 1218-27. Electronic Publication Date: 5 Jun 2009
Journal code: 101130731. E-ISSN: 1535-9786. L-ISSN: 1535-9786.
Report No.: NLM-PMC2725567.
DOI 10.1128/EC.00367-08
CY United States
DT Journal; Article; (JOURNAL ARTICLE) (RESEARCH SUPPORT, N.I.H., EXTRAMURAL) (RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200911
ED Entered STN: 8 Aug 2009
Last Updated on STN: 11 Nov 2009
Entered Medline: 10 Nov 2009
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 49 There are 49 cited references available in MEDLINE for this document.
AB Treatment of systemic fungal infections is difficult because of the limited number of antimycotic drugs available. Thus, there is an immediate need for simple and innovative systems to assay the contribution of individual genes to fungal pathogenesis. We have developed a pathogenesis assay using Caenorhabditis elegans, an established model host, with Saccharomyces cerevisiae as the invading fungus. We have found that yeast infects nematodes, causing disease and death. Our data indicate that the host produces reactive oxygen species (ROS) in response to fungal infection. Yeast mutants sod1Delta and yap1Delta, which cannot withstand ROS, fail to cause disease, except in bli-3 worms, which carry a mutation in a dual oxidase gene. Chemical inhibition of the NADPH oxidase activity abolishes ROS production in worms exposed to yeast. This pathogenesis assay is useful for conducting systematic, whole-genome

screens to identify fungal virulence factors as alternative targets for drug development and exploration of host responses to fungal infections.

L7 ANSWER 41 OF 184 MEDLINE ® on STN DUPLICATE 34
AN 2009108225 MEDLINE Full-text
DN PubMed ID: 19073887
TI Arsenic toxicity to Saccharomyces cerevisiae is a consequence of inhibition of the TORC1 kinase combined with a chronic stress response.
AU Hosiner Dagmar; Lempiainen Harri; Reiter Wolfgang; Urban Joerg; Loewith Robbie; Ammerer Gustav; Schweyen Rudolf; Shore David; Schuller Christoph
CS Department of Genetics, Max F. Perutz Laboratories, University of Vienna, A-1030 Vienna, Austria.
NC P 19966-B12 (Austria Austrian Science Fund FWF)
SO Molecular biology of the cell, (2009 Feb) Vol. 20, No. 3, pp. 1048-57. Electronic Publication Date: 10 Dec 2008
Journal code: 9201390. E-ISSN: 1939-4586. L-ISSN: 1059-1524.
Report No.: NLM-PMC2633375.
DOI 10.1091/mbc.E08-04-0438
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200904
ED Entered STN: 3 Feb 2009
Last Updated on STN: 12 Apr 2009
Entered Medline: 10 Apr 2009
OSC.G 3 There are 3 MEDLINE records that cite this record
REM.CNT 64 There are 64 cited references available in MEDLINE for this document.
AB The conserved Target Of Rapamycin (TOR) growth control signaling pathway is a major regulator of genes required for protein synthesis. The ubiquitous toxic metalloid arsenic, as well as mercury and nickel, are shown here to efficiently inhibit the rapamycin-sensitive TORC1 (TOR complex 1) protein kinase. This rapid inhibition of the TORC1 kinase is demonstrated in vivo by the dephosphorylation and inactivation of its downstream effector, the yeast S6 kinase homolog Sch9. Arsenic, mercury, and nickel cause reduction of transcription of ribosome biogenesis genes, which are under the control of Sfp1, a TORC1-regulated transcriptional activator. We report that arsenic stress deactivates Sfp1 as it becomes dephosphorylated, dissociates from chromatin, and exits the nucleus. Curiously, whereas loss of SFP1 function leads to increased arsenic resistance, absence of TOR1 or SCH9 has the opposite effect suggesting that TORC1 has a role beyond down-regulation of Sfp1. Indeed, we show that arsenic activates the transcription factors Msn2 and Msn4 both of which are targets of TORC1 and protein kinase A (PKA). In contrast to TORC1, PKA activity is not repressed during acute arsenic stress. A normal level of PKA activity might serve to dampen the stress response since hyperactive Msn2 will decrease arsenic tolerance. Thus arsenic toxicity in yeast might be determined by the balance between chronic activation of general stress factors in combination with lowered TORC1 kinase activity.

L7 ANSWER 42 OF 184 MEDLINE ® on STN DUPLICATE 35
AN 2009627199 MEDLINE Full-text
DN PubMed ID: 19184166
TI Use of sugar cane vinasse to mitigate aluminum toxicity to Saccharomyces cerevisiae.
AU de Souza Oliveira Ricardo Pinheiro; Rivas Torres Beatriz; Zilli Mario; de Araujo Viana Marques Daniela; Basso Luiz Carlos; Converti Attilio
CS Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Sao Paulo University, Av Prof Lineu Prestes, 580, Bl. 16, 05508-900 Sao Paulo, Brazil.
SO Archives of environmental contamination and toxicology, (2009 Oct) Vol. 57, No. 3, pp. 488-94. Electronic Publication Date: 29 Jan 2009
Journal code: 0357245. E-ISSN: 1432-0703. L-ISSN: 0090-4341.
DOI 10.1007/s00244-009-9287-x
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 201003
ED Entered STN: 18 Sep 2009
Last Updated on STN: 7 Mar 2010
Entered Medline: 5 Mar 2010
AB Owing to its toxicity, aluminum (Al), which is one of the most abundant metals, inhibits the productivity of many cultures and affects the microbial metabolism. The aim of this work was to investigate the capacity of sugar cane vinasse to mitigate the adverse effects of Al on cell growth, viability, and budding, as the likely result of possible chelating action. For this purpose, Fleischmann's yeast (Saccharomyces cerevisiae) was used in growth tests performed in 125-mL Erlenmeyer flasks containing 30 mL of YED medium (5.0 g/L yeast extract plus 20 g/L glucose) supplemented with the selected amounts of either vinasse or Al in the form of AlCl(3) . H(2)O. Without vinasse, the addition of increasing levels of Al up to 54 mg/L reduced the specific growth rate by 18%, whereas no significant reduction was observed in its presence. The toxic effect of Al on S. cerevisiae growth and the mitigating effect of sugar cane vinasse were quantified by the exponential model of Ciftci et al. (Biotechnol Bioeng 25:2007-2023, 1983). The cell viability decreased from 97.7% at the start to 84.0% at the end of runs without vinasse and to 92.3% with vinasse. On the other hand, the cell budding increased from 7.62% at the start to 8.84% at the end of runs without vinasse and to 17.8% with vinasse. These results demonstrate the ability of this raw material to stimulate cell growth and mitigate the toxic effect of Al.

L7 ANSWER 43 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 36
AN 2011:62086 BIOSIS Full-text
DN PREV201100062086
TI Possible Involvement of Plasmids in the Expression of Killer Toxins of Saccharomyces cerevisiae.
AU Alshalchi, Sahar A. [Reprint Author]; Alrikabi, Sijal W.; Sahi, Ahmad
CS Care of Alshalchi F, Stenhagsvagen 131, S-75260 Uppsala, Sweden
SO Journal of Pure and Applied Microbiology, (OCT 2009) Vol. 3, No. 2, pp. 453-456.
ISSN: 0973-7510.

DT Article
LA English
ED Entered STN: 19 Jan 2011
Last Updated on STN: 19 Jan 2011
AB Four isolates of Saccharomyces cerevisiae were selected in this study for their high ability to produce killer toxin, to investigate possible correlation between their plasmids contents and killer activity Plasmid's profiles on agarose gel revealed presence of two plasmids in each of four isolates Yeast cells obtained after plasmid curing experiments at elevated temperature showed that cured isolates lost their killing capacity The plasmid cured yeast cells originated from three yeast isolates (Scs7, Scvi8 and Scf14) showed the possible correlation between plasmid curing and loss of killing ability, whereas isolate Scf4 retained the killing ability in absence of plasmids Moreover, the extracted plasmid samples were shown to be sensitive to RNase treatment These results might suggest that the genetic determinants for toxin production m three isolates (Scs7, Scvi8 and Scf14) of selected killer's yeasts of S cerevisiae are encoded by dsRNA plasmid(s) except one isolate (Scf4), in this isolate the killer protein may be mediated by chromosome

L7 ANSWER 44 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2009:581187 BIOSIS Full-text
DN PREV200900582290
TI Mycotoxin Toxicity in Saccharomyces cerevisiae Differs Depending on Gene Mutations.
AU Suzuki, Tadahiro; Sirisattha, Sophon; Mori, Katsumi; Iwahashi, Yumiko [Reprint Author]
CS Natl Food Res Inst, Appl Microbiol Div, 2-1-12 Kan Nondai, Tsukuba, Ibaraki 3058642, Japan
suzut@affrc.go.jp
SO Food Science and Technology Research, (JUL 2009) Vol. 15, No. 4, pp. 453-458.
ISSN: 1344-6606.
DT Article
LA English
ED Entered STN: 21 Oct 2009
Last Updated on STN: 21 Oct 2009
AB Mycotoxin inhibits the growth of Saccharomyces cerevisiae mutants with disrupted stress response genes, but no that of wild type yeast even at high concentrations. The S. cerevisiae genome database (SGD) project has recently generated many gene disruptants, including those with disrupted stress response-associated genes. The present study examined the relationship between mycotoxicity and the disruption of functional yeast genes, especially those involved with stress responses. Growth tests showed a major impact in strains other than those with disrupted stress response genes as well as mycotoxin susceptibility of several strains by sodium dodecyl sulfate.

L7 ANSWER 45 OF 184 MEDLINE ® on STN DUPLICATE 37
AN 2009680507 MEDLINE Full-text
DN PubMed ID: 19817264
TI Efficacy and safety of Saccharomyces boulardii in amebiasis-associated diarrhea in children.
AU Savas-Erdeve Senay; Gokay Songul; Dallar Yildiz
CS Department of Pediatrics, Ankara Training and Research Hospital, Ankara, Turkey.
SO The Turkish journal of pediatrics, (2009 May-Jun) Vol. 51, No. 3, pp. 220-4.
Journal code: 0417505. ISSN: 0041-4301. L-ISSN: 0041-4301.
CY Turkey
DT Journal; Article; (JOURNAL ARTICLE)
(RANDOMIZED CONTROLLED TRIAL)
(CLINICAL TRIAL)
LA English
FS MEDLINE; Priority Journals
EM 200910
ED Entered STN: 13 Oct 2009
Last Updated on STN: 30 Oct 2009
Entered Medline: 29 Oct 2009
AB The efficacy and safety of adding Saccharomyces boulardii to antibiotic treatment for amebiasis-associated acute diarrhea in children were assessed in this study. Forty-five children in Group I received only metronidazole per oral for 10 days while 40 patients in Group II received S. boulardii in addition to the same medication. The major outcomes investigated were duration of acute and bloody diarrhea, frequency and consistency of stools, resolution time of the symptoms, and the tolerance and side effects of the treatment regimens. The median duration of acute diarrhea was 5 (1-10) days in Group I and 4.5 (1-10) days in Group II (p=0.965). The median number of stools on follow-up and duration of bloody diarrhea, fever, abdominal pain and vomiting were similar in the two groups. S. boulardii was well tolerated by the children and no side effects were recorded. Addition of S. boulardii to antibiotic treatment of amebiasis-associated acute diarrhea in children does not seem to be more effective than metronidazole treatment alone.

L7 ANSWER 46 OF 184 MEDLINE ® on STN DUPLICATE 38
AN 2009307328 MEDLINE Full-text
DN PubMed ID: 19345671
TI Cd2+, Mn2+, Ni2+ and Se2+ toxicity to Saccharomyces cerevisiae lacking YPK9p the orthologue of human ATP13A2.
AU Schmidt Karyn; Wolfe Devin M; Stiller Barbara; Pearce David A
CS Center for Neural Development and Disease, University of Rochester School of Medicine and Dentistry, Rochester, NY 14620, USA.
NC P30 ES01247 (United States NIEHS NIH HHS)
R01 NS36610 (United States NINDS NIH HHS)
SO Biochemical and biophysical research communications, (2009 May 29) Vol. 383, No. 2, pp. 198-202. Electronic Publication Date: 5 Apr 2009
Journal code: 0372516. E-ISSN: 1090-2104. L-ISSN: 0006-291X.
DOI 10.1016/j.bbrc.2009.03.151
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
LA English
FS MEDLINE; Priority Journals
EM 200905
ED Entered STN: 30 Apr 2009

Last Updated on STN: 20 May 2009
Entered Medline: 19 May 2009
OSC.G 5 There are 5 MEDLINE records that cite this record
AB The Saccharomyces cerevisiae gene YPK9 encodes a putative integral membrane protein which is 58% similar and 38% identical in amino acid sequence to the human lysosomal P(5B) ATPase ATP13A2. Mutations in ATP13A2 have been found in patients with Kufor-Rakeb syndrome, a form of juvenile Parkinsonism. We report that Ypk9p localizes to the yeast vacuole and that deletion of YPK9 confers sensitivity for growth for cadmium, manganese, nickel or selenium. These results suggest that Ypk9p may play a role in sequestration of divalent heavy metal ions. Further studies on the function of Ypk9p/ATP13A2 may help to define the molecular basis of Kufor-Rakeb syndrome and provide a potential link to environmental factors such as heavy metals contributing to some forms of Parkinsonism.

L7 ANSWER 47 OF 184 MEDLINE ® on STN DUPLICATE 39
AN 2008682213 MEDLINE Full-text
DN PubMed ID: 18710911
TI Genomewide expression profiling of cryptolepine-induced toxicity in Saccharomyces cerevisiae.
AU Rojas Marta; Wright Colin W; Pina Benjamin; Portugal Jose
CS Instituto de Biologia Molecular de Barcelona, CSIC, Parc Cientific de Barcelona, Barcelona, Spain.
SO Antimicrobial agents and chemotherapy, (2008 Nov) Vol. 52, No. 11, pp. 3844-50. Electronic Publication Date: 18 Aug 2008
Journal code: 0315061. E-ISSN: 1098-6596. L-ISSN: 0066-4804.
Report No.: NLM-PMC2573129.
DOI 10.1128/AAC.00532-08
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200901
ED Entered STN: 25 Oct 2008
Last Updated on STN: 22 Jan 2009
Entered Medline: 21 Jan 2009
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 29 There are 29 cited references available in MEDLINE for this document.
AB We have used the budding yeast Saccharomyces cerevisiae to identify genes that may confer sensitivity in vivo to the antimalarial and cytotoxic agent cryptolepine. Five S. cerevisiae strains, with different genetic backgrounds in cell permeability and DNA damage repair mechanisms, were exposed to several concentrations of cryptolepine. Cryptolepine showed a relatively mild toxicity for wild-type strains, which was augmented by either increasing cell permeability (Deltaerg6 or ISE2 strains) or disrupting DNA damage repair (Deltarad52 strains). These results are compatible with the ability of cryptolepine to intercalate into DNA and thus promote DNA lesions. The effects of low concentrations of cryptolepine (20% and 40% inhibitory concentrations [IC(20) and IC(40)]) were analyzed by comparing the gene expression profiles of treated and untreated Deltaerg6 yeast cells. Significant changes in expression levels were observed for 349 genes (117 upregulated and 232 downregulated). General stress-related genes constituted the only recognizable functional cluster whose expression was increased upon cryptolepine treatment, making up about 20% of upregulated genes. In contrast, analysis of the characteristics of downregulated genes revealed a specific effect of cryptolepine on genes related to iron transport or acid phosphatases, as well as a significant proportion of genes related to cell wall components. The effects of cryptolepine on the transcription of iron transport-related genes were consistent with a loss of function of the iron sensor Aft1p, indicating a possible disruption of iron metabolism in S. cerevisiae. Since the interference of cryptolepine with iron metabolism is considered one of its putative antimalarial targets, this finding supports the utility of S. cerevisiae in drug-developing schemes.

L7 ANSWER 48 OF 184 MEDLINE ® on STN DUPLICATE 40
AN 2008525375 MEDLINE Full-text
DN PubMed ID: 18579505
TI Interplay of DNA repair pathways controls methylation damage toxicity in Saccharomyces cerevisiae.
AU Cejka Petr; Jiricny Josef
CS Institute of Molecular Cancer Research, University of Zurich, CH-8057 Zurich, Switzerland.
SO Genetics, (2008 Aug) Vol. 179, No. 4, pp. 1835-44. Electronic Publication Date: 24 Jun 2008
Journal code: 0374636. ISSN: 0016-6731. L-ISSN: 0016-6731.
Report No.: NLM-PMC2516062.
DOI 10.1534/genetics.108.089979
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200811
ED Entered STN: 20 Aug 2008
Last Updated on STN: 13 Nov 2008
Entered Medline: 12 Nov 2008
OSC.G 3 There are 3 MEDLINE records that cite this record
REM.CNT 44 There are 44 cited references available in MEDLINE for this document.
AB Methylating agents of S(N)1 type are widely used in cancer chemotherapy, but their mode of action is poorly understood. In particular, it is unclear how the primary cytotoxic lesion, O(6)-methylguanine ((Me)G), causes cell death. One hypothesis stipulates that binding of mismatch repair (MMR) proteins to (Me)G/T mispairs arising during DNA replication triggers cell-cycle arrest and cell death. An alternative hypothesis posits that (Me)G cytotoxicity is linked to futile processing of (Me)G-containing base pairs by the MMR system. In this study, we provide compelling genetic evidence in support of the latter hypothesis. Treatment of 4644 deletion mutants of Saccharomyces cerevisiae with the prototypic S(N)1-type methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) identified MMR as the only pathway that sensitizes cells to MNNG. In contrast, homologous recombination (HR), postreplicative repair, DNA helicases, and chromatin maintenance factors protect yeast cells against the cytotoxicity of this chemical. Notably, DNA damage signaling proteins played a protective rather than sensitizing role in the MNNG response. Taken together, this evidence demonstrates that (Me)G-containing lesions in yeast must be processed to be cytotoxic.

L7 ANSWER 49 OF 184 MEDLINE ® on STN DUPLICATE 41
AN 2008782925 MEDLINE Full-text

DN PubMed ID: 18811659
TI Overexpression of the aldose reductase GRE3 suppresses lithium-induced galactose toxicity in Saccharomyces cerevisiae.
AU Masuda Claudio A; Previato Jose O; Miranda Michel N; Assis Leandro J; Penha Luciana L; Mendonca-Previato Lucia; Montero-Lomeli Monica
CS Instituto de Bioquimica Medica, Programa de Biotecnologia e Biologia Molecular, Centro de Ciencias da Saude, Universidade Federal do Rio de Janeiro, Brazil.
SO FEMS yeast research, (2008 Dec) Vol. 8, No. 8, pp. 1245-53. Electronic Publication Date: 22 Sep 2008
Journal code: 101085384. ISSN: 1567-1356. L-ISSN: 1567-1356.
DOI 10.1111/j.1567-1364.2008.00440.x
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200901
ED Entered STN: 5 Dec 2008
Last Updated on STN: 13 Jan 2009
Entered Medline: 12 Jan 2009
AB In Saccharomyces cerevisiae, lithium induces a 'galactosemia-like' phenotype as a consequence of inhibition of phosphoglucomutase, a key enzyme in galactose metabolism. Induced galactose toxicity is prevented by deletion of GAL4, which inhibits the transcriptional activation of genes involved in galactose metabolism and by deletion of the galactokinase (GAL1), indicating that galactose-1-phosphate, a phosphorylated intermediate of the Leloir pathway, is the toxic compound. As an alternative to inhibiting entry and metabolism of galactose, we investigated whether deviation of galactose metabolism from the Leloir pathway would also overcome the galactosemic effect of lithium. We show that cells overexpressing the aldose reductase GRE3, which converts galactose to galactitol, are more tolerant to lithium than wild-type cells when grown in galactose medium and they accumulate more galactitol and less galactose-1-phosphate. Overexpression of GRE3 also suppressed the galactose growth defect of the 'galactosemic'gal7- and gal10-deleted strains, which lack galactose-1-P-uridyltransferase or UDP-galactose-4-epimerase activities, respectively. Furthermore, the effect of GRE3 was independent of the inositol monophosphatases INM1 and INM2. We propose that lithium induces a galactosemic state in yeast and that inhibition of the Leloir pathway before the phosphorylation step or stimulation of galactitol production suppresses lithium-induced galactose toxicity.

L7 ANSWER 50 OF 184 MEDLINE ® on STN DUPLICATE 42
AN 2008179581 MEDLINE Full-text
DN PubMed ID: 18194258
TI Extracellular methylglyoxal toxicity in Saccharomyces cerevisiae: role of glucose and phosphate ions.
AU Ispolnov K; Gomes R A; Silva M S; Freire A P
CS Centro de Quimica e Bioquimica, Departamento de Quimica e Bioquimica, Faculdade de Ciencias da Universidade de Lisboa, Lisboa, Portugal.
SO Journal of applied microbiology, (2008 Apr) Vol. 104, No. 4, pp. 1092-102. Electronic Publication Date: 7 Jan 2008
Journal code: 9706280. E-ISSN: 1365-2672. L-ISSN: 1364-5072.
DOI 10.1111/j.1365-2672.2007.03641.x
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200807
ED Entered STN: 15 Mar 2008
Last Updated on STN: 22 Jul 2008
Entered Medline: 21 Jul 2008
OSC.G 1 There are 1 MEDLINE records that cite this record
AB AIM: The purpose of this study was to investigate the behaviour of Saccharomyces cerevisiae in response to extracellular methylglyoxal.

METHODS AND RESULTS: Cell survival to methylglyoxal and the importance of phosphates was investigated. The role of methylglyoxal detoxification systems and methylglyoxal-derived protein glycation were studied and the relation to cell survival or death was evaluated. Extracellular methylglyoxal decreased cell viability, and the presence of phosphate enhanced this effect. D-glucose seems to exert a protective effect towards this toxicity. Methylglyoxal-induced cell death was not apoptotic and was not related to intracellular glycation processes. The glyoxalases and aldose reductase were important in methylglyoxal detoxification. Mutants lacking glyoxalase I and II showed increased sensitivity to methylglyoxal, while strains overexpressing these genes had increased resistance.

CONCLUSIONS: Extracellular methylglyoxal induced non-apoptotic cell death, being unrelated to glycation. Inactivation of methylglyoxal-detoxifying enzymes by phosphate is one probable cause. Phosphate and D-glucose may also act through their complex involvement in stress response mechanisms.

SIGNIFICANCE AND IMPACT OF THE STUDY: These findings contribute to elucidate the mechanisms of cell toxicity by methylglyoxal. This information could be useful to on-going studies using yeast as a eukaryotic cell model to investigate methylglyoxal-derived glycation and its role in neurodegenerative diseases.

L7 ANSWER 51 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 43
AN 2008:675452 BIOSIS Full-text
DN PREV200800675451
TI Exploring predictive QSAR models using Quantum Topological Molecular Similarity (QTMS) descriptors for toxicity of nitroaromatics to Saccharomyces cerevisiae.
AU Roy, Kunal [Reprint Author]; Popelier, Paul L. A.
CS Manchester Interdisciplinary Bioctr, 131 Princess St, Manchester M1 7DN, Lancs, UK
kroy@pharma.jdvu.ac.in; pla@manchester.ac.uk
SO QSAR & Combinatorial Science, (AUG 2008) Vol. 27, No. 8, pp. 1006-1012. ISSN: 1611-020X.
DT Article

LA English
ED Entered STN: 27 Nov 2008
Last Updated on STN: 27 Nov 2008
AB In view of the widespread industrial use of nitroaromatics and their consequent ecotoxicological hazard potential, we constructed predictive Quantitative Structure-Activity Relationship (QSAR) models for the toxicity of nitroaromatics to the ecologically important species Saccharomyces cerevisiae. We used Quantum Topological Molecular Similarity (QTMS) descriptors along with electrophilicity index (E-LUMO) and lipid water partition coefficient (log K-ow) as predictor variables. The QTMS descriptors were calculated at B3LYP/6-311 + G(2d,p) level of theory. QTMS descriptors were employed to complement the deficiency of E-LUMO in setting up predictive QSAR models from the view point of external validation. The dataset was divided into a training set (18 compounds) and test set (six compounds) in a ratio of three to one. Partial Least Square (PLS) models were developed based on the training set compounds. The predictive capacity of the models was assessed by the test compounds. The models were also validated by a randomisation test and leave-one-seventh-out crossvalidation test. The results suggest that Bond Critical Point (BCP) descriptors can develop predictive QSAR models for nitroaromatic toxicity to Saccharomyces cerevisiae when used along with E-LUMO and log K-OW, The diagnostic potential of QTMS descriptors could also reveal the importance of the nitro group for nitroaromatic toxicity.

L7 ANSWER 52 OF 184 MEDLINE ® on STN DUPLICATE 44
AN 2008584932 MEDLINE Full-text
DN PubMed ID: 18528910
TI Mechanisms of copper toxicity in Saccharomyces cerevisiae determined by microarray analysis.
AU Yasokawa Daisuke; Murata Satomi; Kitagawa Emiko; Iwahashi Yumiko; Nakagawa Ryoji; Hashido Tazusa; Iwahashi Hitoshi
CS Hokkaido Food Processing Research Center, 589-4 Bunkyodai Midorimachi, Ebetsu, Hokkaido 0690836, Japan. duke@foodhokkaido.gr.jp
SO Environmental toxicology, (2008 Oct) Vol. 23, No. 5, pp. 599-606.
Journal code: 100885357. E-ISSN: 1522-7278. L-ISSN: 1520-4081.
DOI 10.1002/tox.20406
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 200811
ED Entered STN: 9 Sep 2008
Last Updated on STN: 31 Dec 2008
Entered Medline: 24 Nov 2008
AB The effect of the heavy metal copper on the expression of a wide spectrum of genes was analyzed by using a DNA microarray. The gene expression profile of baker's yeast Saccharomyces cerevisiae grown in a medium containing a sublethal concentration of cupric sulfate was compared with that of yeast grown in a normal medium. Among approximately 6000 yeast ORFs, 143 ORFs were induced more than twofold to resist copper toxicity after exposure to copper. Copper metallothionein CUP1-1 and CUP1-2 were induced more than 20-fold. Some genes related to sulfur metabolism and oxidative stress response were also up-regulated. This DNA microarray analysis identified several molecular targets of copper toxicity.

L7 ANSWER 53 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 45
AN 2009:125861 BIOSIS Full-text
DN PREV200900125861
TI Heterologous Expression of Cholera Toxin B Subunit in Saccharomyces cerevisiae.
AU Lim, Jung-Gu; Jin, Hyo-Sang [Reprint Author]
CS Jeonju Univ, Coll Alternat Med, Jeonju 560759, South Korea
jin@jj.ac.kr
SO Biotechnology and Bioprocess Engineering, (SEP-OCT 2008) Vol. 13, No. 5, pp. 598-605.
ISSN: 1226-8372.
DT Article
LA English
OS GenBank-CAA41591; EMBL-CAA41591; DDBJ-CAA41591
ED Entered STN: 18 Feb 2009
Last Updated on STN: 18 Feb 2009
AB The cholera toxin B subunit (CTB), which consists of five identical polypeptides and adopts a pentameric structure, has been shown to bind to the GM1-gangliosides at the cellular surface. Recombinant CTB has attracted much attention due to its non-toxicity and potential as a strong immunogenic antigen and immuno adjuvant for both system and mucosal immune responses. In this study, CTB was expressed in Saccharomyces cerevisiae and the resulting recombinant CTB was extensively characterized. PCR and back-transformation into E coli confirmed the presence of the CTB gene-containing plasmid in the transformants and northern analysis showed the presence of the CTB-specific transcript. Western blot analysis of the yeast-derived protein extract showed the presence of CTB with mobility similar to purified CTB from Vibrio cholerae suggesting that the expressed CTB assembled into the desired pentameric form. Quantitative ELISA revealed that the recombinant CTB comprised approximately 0.5 similar to 1.3% of the total cell-free extract. In addition, 0.5 similar to 2 mg of CTB protein per liter of cultured media was detected 1 day, at the earliest after cultivation. The GM1-ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) confirmed that the yeast-derived CTB bound specifically to the GM1-ganglioside receptor, indicating that it retained its native function and pentameric form, which is required for binding to intestinal epithelial cell membrane glycolipid receptors. In addition to the development of a yeast-derived edible vaccine against cholera, this study regarding the expression and assembly of recombinant CTB into biologically active oligomers in recombinant S. cerevisiae enables the efficient production of a GRAS microorganism-based adjuvant, as well as the development of carriers for foreign vaccine molecules. (C) KSBB

L7 ANSWER 54 OF 184 MEDLINE ® on STN DUPLICATE 46
AN 2008568750 MEDLINE Full-text
DN PubMed ID: 18421797
TI The roles of galactitol, galactose-1-phosphate, and phosphoglucomutase in galactose-induced toxicity in Saccharomyces cerevisiae.
AU de Jongh Willem A; Bro Christoffer; Ostergaard Simon; Regenberg Birgitte; Olsson Lisbeth; Nielsen Jens
CS Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, Building 223, Soltofts Plads, DK-2800 Kgs. Lyngby, Denmark.
SO Biotechnology and bioengineering, (2008 Oct 1) Vol. 101, No. 2, pp. 317-26.
Journal code: 7502021. E-ISSN: 1097-0290. L-ISSN: 0006-3592.
DOI 10.1002/bit.21890

CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200809
ED Entered STN: 3 Sep 2008
Last Updated on STN: 17 Sep 2008
Entered Medline: 16 Sep 2008
OSC.G 5 There are 5 MEDLINE records that cite this record
AB The uptake and catabolism of galactose by the yeast Saccharomyces cerevisiae is much lower than for glucose and fructose, and in applications of this yeast for utilization of complex substrates that contain galactose, for example, lignocellulose and raffinose, this causes prolonged fermentations. Galactose is metabolized via the Leloir pathway, and besides the industrial interest in improving the flux through this pathway it is also of medical relevance to study the Leloir pathway. Thus, genetic disorders in the genes encoding galactose-1-phosphate uridylyltransferase or galactokinase result in galactose toxicity both in patients with galactosemia and in yeast. In order to elucidate galactose related toxicity, which may explain the low uptake and catabolic rates of S. cerevisiae, we have studied the physiological characteristics and intracellular metabolite profiles of recombinant S. cerevisiae strains with improved or impaired growth on galactose. Aerobic batch cultivations on galactose of strains with different combinations of overexpression of the genes GAL1, GAL2, GAL7, and GAL10, which encode proteins that together convert extracellular galactose into glucose-1-phosphate, revealed a decrease in the maximum specific growth rate when compared to the reference strain. The hypothesized toxic intermediate galactose-1-phosphate cannot be the sole cause of galactose related toxicity, but indications were found that galactose-1-phosphate might cause a negative effect through inhibition of phosphoglucomutase. Furthermore, we show that galactitol is formed in S. cerevisiae, and that the combination of elevated intracellular galactitol concentration, and the ratio between galactose-1-phosphate concentration and phosphoglucomutase activity seems to be important for galactose related toxicity causing decreased growth rates.

L7 ANSWER 55 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2008:182614 BIOSIS Full-text
DN PREV200800186719
TI Identification of genes involved in the toxic response of Saccharomyces cerevisiae against iron and copper overload by parallel analysis of deletion mutants (vol 101, pg 140, 2008).
AU Jo, William J. [Reprint Author]; Loguinov, Alex; Chang, Michelle; Wintz, Henri; Nislow, Corey; Arkin, Adam P.; Giaever, Guri; Vulpe, Chris D.
CS Univ Calif Berkeley, Berkeley, CA 94720 USA
SO Toxicological Sciences, (MAR 2008) Vol. 102, No. 1, pp. 205.
ISSN: 1096-6080.
DT Article
Errata
LA English
ED Entered STN: 12 Mar 2008
Last Updated on STN: 12 Mar 2008

L7 ANSWER 56 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 2008082072 EMBASE Full-text
TI Erratum: Identification of genes involved in the toxic response of saccharomyces cerevisiae against iron and copper overload by parallel analysis of deletion mutants (Toxicological Sciences (2008) volume 101 (140-151) 10.1093/toxsci/kfm226).
AU Jo, William J.; Loguinov, Alex; Chang, Michelle; Wintz, Henri; Nislow, Corey; Arkin, Adam P.; Giaever, Guri; Vulpe, Chris D.
SO Toxicological Sciences, (Mar 2008) Vol. 102, No. 1, pp. 205.
ISSN: 1096-6080; E-ISSN: 1096-0929 CODEN: TOSCF2
CY United States
DT Journal; Errata; (Erratum)
FS 052 Toxicology
LA English
ED Entered STN: 27 Feb 2008
Last Updated on STN: 27 Feb 2008

L7 ANSWER 57 OF 184 MEDLINE ® on STN DUPLICATE 47
AN 2007716160 MEDLINE Full-text
DN PubMed ID: 17785683
TI Identification of genes involved in the toxic response of Saccharomyces cerevisiae against iron and copper overload by parallel analysis of deletion mutants.
AU Jo William J; Loguinov Alex; Chang Michelle; Wintz Henri; Nislow Corey; Arkin Adam P; Giaever Guri; Vulpe Chris D
CS Department of Nutritional Sciences and Toxicology, Superfund Basic Research Program [corrected] University of California Berkeley, Berkeley, California 94720, USA.
SO Toxicological sciences : an official journal of the Society of Toxicology, (2008 Jan) Vol. 101, No. 1, pp. 140-51. Electronic Publication Date: 4 Sep 2007
Journal code: 9805461. ISSN: 1096-6080. L-ISSN: 1096-0929.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 200802
ED Entered STN: 11 Dec 2007
Last Updated on STN: 21 Feb 2008
Entered Medline: 20 Feb 2008
OSC.G 12 There are 12 MEDLINE records that cite this record
AB Iron and copper are essential nutrients for life as they are required for the function of many proteins but can be toxic if present in excess. Accumulation of these metals in the human body as a consequence of overload disorders and/or high environmental exposures has detrimental effects on health. The budding yeast Saccharomyces cerevisiae is an accepted cellular model for iron and copper metabolism in humans primarily because of the high degree of conservation between pathways and proteins involved. Here we report a systematic screen using yeast deletion mutants to identify genes involved in the toxic response to growth-inhibitory concentrations of iron and copper

sulfate. We aimed to understand the cellular responses to toxic concentrations of these two metals by analyzing the different subnetworks and biological processes significantly enriched with these genes. Our results indicate the presence of two different detoxification pathways for iron and copper that converge toward the vacuole. The product of several of the identified genes in these pathways form molecular complexes that are conserved in mammals and include the retromer, endosomal sorting complex required for transport (ESCRT) and AP-3 complexes, suggesting that the mechanisms involved can be extrapolated to humans. Our data also suggest a disruption in ion homeostasis and, in particular, of iron after copper exposure. Moreover, the identification of treatment-specific genes associated with biological processes such as DNA double-strand break repair for iron and tryptophan biosynthesis for copper suggests differences in the mechanisms by which these two metals are toxic at high concentrations.

L7 ANSWER 58 OF 184 MEDLINE ® on STN DUPLICATE 48
AN 2007166127 MEDLINE Full-text
DN PubMed ID: 17261587
TI Extracellular production of hydrogen selenide accounts for thiol-assisted toxicity of selenite against Saccharomyces cerevisiae.
AU Tarze Agathe; Dauplais Marc; Grigoras Ioana; Lazard Myriam; Ha-Duong Nguyet-Thanh; Barbier Frederique; Blanquet Sylvain; Plateau Pierre
CS Laboratoire de Biochimie, UMR CNRS 7654, Departement de Biologie, Ecole Polytechnique, 91128 Palaiseau Cedex, France.
SO The Journal of biological chemistry, (2007 Mar 23) Vol. 282, No. 12, pp. 8759-67. Electronic Publication Date: 29 Jan 2007
Journal code: 2985121R. ISSN: 0021-9258. L-ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200705
ED Entered STN: 20 Mar 2007
Last Updated on STN: 3 May 2007
Entered Medline: 2 May 2007
OSC.G 8 There are 8 MEDLINE records that cite this record
AB Administration of selenium in humans has anticarcinogenic effects. However, the boundary between cancer-protecting and toxic levels of selenium is extremely narrow. The mechanisms of selenium toxicity need to be fully understood. In Saccharomyces cerevisiae, selenite in the millimolar range is well tolerated by cells. Here we show that the lethal dose of selenite is reduced to the micromolar range by the presence of thiols in the growth medium. Glutathione and selenite spontaneously react to produce several selenium-containing compounds (selenodiglutathione, glutathioselenol, hydrogen selenide, and elemental selenium) as well as reactive oxygen species. We studied which compounds in the reaction pathway between glutathione and sodium selenite are responsible for this toxicity. Involvement of selenodiglutathione, elemental selenium, or reactive oxygen species could be ruled out. In contrast, extracellular formation of hydrogen selenide can fully explain the exacerbation of selenite toxicity by thiols. Indeed, direct production of hydrogen selenide with D-cysteine desulfhydrase induces high mortality. Selenium uptake by S. cerevisiae is considerably enhanced in the presence of external thiols, most likely through internalization of hydrogen selenide. Finally, we discuss the possibility that selenium exerts its toxicity through consumption of intracellular reduced glutathione, thus leading to severe oxidative stress.

L7 ANSWER 59 OF 184 MEDLINE ® on STN DUPLICATE 49
AN 2007451640 MEDLINE Full-text
DN PubMed ID: 17669103
TI Efficacy and safety of Saccharomyces boulardii in the 14-day triple anti-Helicobacter pylori therapy: a prospective randomized placebo-controlled double-blind study.
AU Cindoruk Mehmet; Erkan Gulbanu; Karakan Tarkan; Dursun Ayse; Unal Selahattin
CS Department of Gastroenterology, School of Medicine, Gazi University, Ankara, Turkey.
SO Helicobacter, (2007 Aug) Vol. 12, No. 4, pp. 309-16.
Journal code: 9605411. ISSN: 1083-4389. L-ISSN: 1083-4389.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RANDOMIZED CONTROLLED TRIAL)
(CLINICAL TRIAL)
LA English
FS MEDLINE; Priority Journals
EM 200712
ED Entered STN: 3 Aug 2007
Last Updated on STN: 14 Dec 2007
Entered Medline: 13 Dec 2007
OSC.G 2 There are 2 MEDLINE records that cite this record
AB BACKGROUND: Recent studies indicate a potential role of Saccharomyces boulardii in the prevention of Helicobacter pylori treatment-related side-effects and also in improvement of eradication rate. Our aim is to investigate the efficacy and safety of S. boulardii in the prevention of side-effects related to H. pylori eradication. The secondary aim of the study was to define the effect of S. boulardii on the eradication success of anti-H. pylori therapy.

MATERIALS AND METHODS: One hundred and twenty-four patients with H. pylori infection (male/female: 44/80, mean age: 48 +/- 14.25 year) receiving 14 days of triple therapy (clarithromycin 500 mg b.i.d., amoxicillin 1000 mg b.i.d., and lansoprazole 30 mg b.i.d.) were randomly assigned to S. boulardii or placebo. Dyspeptic symptoms were recorded by using modified Glasgow Dyspepsia Questionnaire (GDQ). Side-effect profile and tolerability were assessed using a symptom-based questionnaire. H. pylori status was rechecked after 6 weeks after completion of eradication therapy.

RESULTS: H. pylori eradication rate, although higher in the treatment group, was statistically similar in treatment and control groups: 71% (44/62) versus 59.7% (37/62), respectively ($p > .05$). Nine (14.5%) patients in the treatment group and 19 (30.6%) patients in the placebo group experienced diarrhea ($p < .05$). Epigastric discomfort was more frequent in the control group [9 (14.5%) versus 27 (43.5%), respectively ($p < .01$)]. Diffuse abdominal pain, abdominal gas, taste disturbance, urticaria, nausea symptoms were similar in both groups. GDQ scores after treatment were significantly better for treatment group (mean +/- SD, range: 1.38 +/- 1.25 (0-5) vs. 2.22 +/- 1.44 (0-6), respectively; $p < .01$).

CONCLUSION: S. boulardii improved anti-H. pylori antibiotherapy-associated diarrhea, epigastric discomfort, and treatment tolerability. In addition, S. boulardii supplement decreased post-treatment dyspepsia symptoms independent of H. pylori status. However, S. boulardii had no significant affect on the rate of H. pylori eradication.

L7 ANSWER 60 OF 184 MEDLINE ® on STN DUPLICATE 50
AN 2009091189 MEDLINE Full-text
DN PubMed ID: 19164913
TI Protein aggregation and polyasparagine-mediated cellular toxicity in Saccharomyces cerevisiae.
AU Peters Theodore W; Huang Mingxia
CS Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, Colorado 80045, USA.
SO Prion, (2007 Apr-Jun) Vol. 1, No. 2, pp. 144-53. Electronic Publication Date: 26 Apr 2007
Journal code: 101472305. E-ISSN: 1933-690X. L-ISSN: 1933-6896.
Report No.: NLM-PMC2634455.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 200903
ED Entered STN: 24 Jan 2009
Last Updated on STN: 19 Mar 2009
Entered Medline: 18 Mar 2009
OSC.G 3 There are 3 MEDLINE records that cite this record
REM.CNT 74 There are 74 cited references available in MEDLINE for this document.
AB It is well established that protein aggregation is associated with many neurodegenerative disorders including polyglutamine diseases, but a mechanistic understanding of the role of protein aggregates in the disease pathogenesis remains elusive. Previously thought to be the cause of cellular toxicity such as cellular dysfunction and cell death, protein aggregation is now proposed to serve a protective role by sequestering toxic oligomers from interfering with essential physiological processes. To investigate the relationship between protein aggregation and cellular toxicity, we have characterized and compared the effects of two GFP-fusion proteins that form aggregates in Saccharomyces cerevisiae, one with a polyasparagine repeat (GFP(N104)) and one without (GFP(C)). Although both proteins can form microscopically visible GFP-positive aggregates, only the GFP(N104)-containing aggregates exhibit morphological and biochemical characteristics that resemble the aggregates formed by mutant huntingtin in yeast cells. Formation of both the GFP(C) and GFP(N104) aggregates depends on microtubules, while only the GFP(N104) aggregate requires the chaperone Hsp104 and the prion Rnq1 and is resistant to SDS. Although no microscopically visible GFP(N104) aggregates were observed in the hsp104Delta and rnq1Delta mutant cells, SDS-insoluble aggregates can still be detected by the filter trap assay. These observations argue that the GFP(N104)-containing aggregates can exist in at least two distinct states in vivo. We also show that a nucleus-targeted GFP(N104) interferes with transcription from two SAGA-dependant promoters and results in a decrease in cell viability. Overall, the results imply that the GFP(N104) protein behaves similarly to the mutant huntingtin in yeast cells and provides a new model for investigating the interplay between protein aggregates and the associated phenotypes.

L7 ANSWER 61 OF 184 MEDLINE ® on STN DUPLICATE 51
AN 2006400644 MEDLINE Full-text
DN PubMed ID: 16820484
TI Old yellow enzymes protect against acrolein toxicity in the yeast Saccharomyces cerevisiae.
AU Trotter Eleanor W; Collinson Emma J; Dawes Ian W; Grant Chris M
CS The University of Manchester, Faculty of Life Sciences, The Michael Smith Building, Oxford Road, Manchester M13 9PT, United Kingdom.
NC (United Kingdom Wellcome Trust)
SO Applied and environmental microbiology, (2006 Jul) Vol. 72, No. 7, pp. 4885-92.
Journal code: 7605801. ISSN: 0099-2240. L-ISSN: 0099-2240.
Report No.: NLM-PMC1489299.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200609
ED Entered STN: 6 Jul 2006
Last Updated on STN: 6 Sep 2006
Entered Medline: 5 Sep 2006
OSC.G 6 There are 6 MEDLINE records that cite this record
REM.CNT 31 There are 31 cited references available in MEDLINE for this document.
AB Acrolein is a ubiquitous reactive aldehyde which is formed as a product of lipid peroxidation in biological systems. In this present study, we screened the complete set of viable deletion strains in Saccharomyces cerevisiae for sensitivity to acrolein to identify cell functions involved in resistance to reactive aldehydes. We identified 128 mutants whose gene products are localized throughout the cell. Acrolein-sensitive mutants were distributed among most major biological processes but particularly affected gene expression, metabolism, and cellular signaling. Surprisingly, the screen did not identify any antioxidants or similar stress-protective molecules, indicating that acrolein toxicity may not be mediated via reactive oxygen species. Most strikingly, a mutant lacking an old yellow enzyme (OYE2) was identified as being acrolein sensitive. Old yellow enzymes are known to reduce alpha,beta-unsaturated carbonyl compounds in vitro, but their physiological roles have remained uncertain. We show that mutants lacking OYE2, but not OYE3, are sensitive to acrolein, and overexpression of both isoenzymes increases acrolein tolerance. Our data indicate that OYE2 is required for basal levels of tolerance, whereas OYE3 expression is particularly induced following acrolein stress. Despite the range of alpha,beta-unsaturated carbonyl compounds that have been identified as substrates of old yellow enzymes in vitro, we show that old yellow enzymes specifically mediate resistance to small alpha,beta-unsaturated carbonyl compounds, such as acrolein, in vivo.

L7 ANSWER 62 OF 184 MEDLINE ® on STN DUPLICATE 52
AN 2006074776 MEDLINE Full-text
DN PubMed ID: 16461706
TI Use of PMA1 as a housekeeping biomarker for assessment of toxicant-induced stress in Saccharomyces cerevisiae.
AU Schmitt Marcel; Schwanewilm Petra; Ludwig Jost; Lichtenberg-Frate Hella
CS Institut fur Zellulare und Molekulare Botanik, AG Molekulare Bioenergetik,

Universitat Bonn, Kirschallee 1, 53115 Bonn, Germany.
SO Applied and environmental microbiology, (2006 Feb) Vol. 72, No. 2, pp. 1515-22.
Journal code: 7605801. ISSN: 0099-2240. L-ISSN: 0099-2240.
Report No.: NLM-PMC1392943.
CY United States
DT (IN VITRO)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200604
ED Entered STN: 8 Feb 2006
Last Updated on STN: 21 Apr 2006
Entered Medline: 20 Apr 2006
REM.CNT 30 There are 30 cited references available in MEDLINE for this document.
AB The brewer's yeast Saccharomyces cerevisiae has emerged as a versatile and robust model system for laboratory use to study toxic effects of various substances. In this study, toxicant-induced stresses of pure compounds were investigated in Saccharomyces cerevisiae utilizing a destabilized version of the green fluorescent protein optimized for expression in yeast (yEGFP3) under control of the promoter of the housekeeping plasma membrane ATPase gene PMA1. The responses of the biomarker upon increasing test compound concentrations were monitored by determining the decrease in fluorescence. The reporter assay deployed a simple and robust protocol for the rapid detection of toxic effects within a 96-well microplate format. Fluorescence emissions were normalized to cell growth determined by absorption and were correlated to internal reference standards. The results were expressed as effective concentrations (EC20). Dose-response experiments were conducted in which yeast cells were exposed in minimal medium and in the presence of 20% fetal calf serum to sublethal concentrations of an array of heavy metals, salt, and a number of stress-inducing compounds (Diclofenac, Lindane, methyl-N-nitro-N-nitrosoguanidine [MNNG], hydroxyurea, and caffeine). Long-term exposure (7 h) played a considerable role in the adaptive response to intoxication compared to early responses at 4 h exposure. The data obtained after 4 h of exposure and expressed as EC20 were compared to 50% inhibitory concentration values derived from cell line and ecotoxicological tests. This study demonstrates the versatility of the novel biomarker to complement existing test batteries to assess contaminant exposure and effects.

L7 ANSWER 63 OF 184 MEDLINE ® on STN DUPLICATE 53
AN 2006563035 MEDLINE Full-text
DN PubMed ID: 16956380
TI Role of protein phosphatases 2C on tolerance to lithium toxicity in the yeast Saccharomyces cerevisiae.
AU Ruiz Amparo; Gonzalez Asier; Garcia-Salcedo Raul; Ramos Jose; Arino Joaquin
CS Departament de Bioquimica i Biologia Molecular, Universitat Autonoma de Barcelona, Bellaterra 08193, Barcelona, Spain.
SO Molecular microbiology, (2006 Oct) Vol. 62, No. 1, pp. 263-77. Electronic Publication Date: 31 Aug 2006
Journal code: 8712028. ISSN: 0950-382X. L-ISSN: 0950-382X.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200701
ED Entered STN: 22 Sep 2006
Last Updated on STN: 24 Jan 2007
Entered Medline: 23 Jan 2007
OSC.G 6 There are 6 MEDLINE records that cite this record
AB Protein phosphatases 2C are a family of conserved enzymes involved in many aspects of the cell biology. We reported that, in the yeast Saccharomyces cerevisiae, overexpression of the Ptc3p isoform resulted in increased lithium tolerance in the hypersensitive hal3 background. We have found that the tolerance induced by PTC3 overexpression is also observed in wild-type cells and that this is most probably the result of increased expression of the ENA1 Na(+)-ATPase mediated by the Hog1 MAP kinase pathway. This effect does not require a catalytically active protein. Surprisingly, deletion of PTC3 (similarly to that of PTC2, PTC4 or PTC5) does not confer a lithium-sensitive phenotype, but mutation of PTC1 does. Lack of PTC1 in an ena1-4 background did not result in additive lithium sensitivity and the ptc1 mutant showed a decreased expression of the ENA1 gene in cells stressed with LiCl. In agreement, under these conditions, the ptc1 mutant was less effective in extruding Li(+) and accumulated higher concentrations of this cation. Deletion of PTC1 in a hal3 background did not exacerbate the halosensitive phenotype of the hal3 strain. In addition, induction from the ENA1 promoter under LiCl stress decreased similarly (50%) in hal3, ptc1 and ptc1 hal3 mutants. Finally, mutation of PTC1 virtually abolishes the increased tolerance to toxic cations provided by overexpression of Hal3p. These results indicate that Ptc1p modulates the function of Ena1p by regulating the Hal3/Ppz1,2 pathway. In conclusion, overexpression of PTC3 and lack of PTC1 affect lithium tolerance in yeast, although through different mechanisms.

L7 ANSWER 64 OF 184 MEDLINE ® on STN DUPLICATE 54
AN 2006041469 MEDLINE Full-text
DN PubMed ID: 16292090
TI Efficacy and safety of Saccharomyces boulardii in prevention of antibiotic-associated diarrhoea due to Helicobacterpylori eradication.
AU Duman Deniz Guney; Bor Serhat; Ozutemiz Omer; Sahin Tulin; Oguz Dilek; Istan Fahri; Vural Tumer; Sandkci Macit; Isksal Fatih; Simsek Ilkay; Soyturk Mujde; Arslan Serap; Sivri Bulent; Soykan Irfan; Temizkan Aysegul; Bessk Fatih; Kaymakoglu Sabahattin; Kalayc Cem
CS Department of Gastroenterology, Marmara University, School of Medicine, Istanbul, Turkey.
SO European journal of gastroenterology & hepatology, (2005 Dec) Vol. 17, No. 12, pp. 1357-61.
Journal code: 9000874. ISSN: 0954-691X. L-ISSN: 0954-691X.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(MULTICENTER STUDY)
(RANDOMIZED CONTROLLED TRIAL)
(CLINICAL TRIAL)
LA English
FS MEDLINE; Priority Journals
EM 200606

ED Entered STN: 25 Jan 2006
Last Updated on STN: 30 Jun 2006
Entered Medline: 29 Jun 2006
OSC.G 4 There are 4 MEDLINE records that cite this record
AB BACKGROUND AND AIM: Antibiotic-associated diarrhoea may develop during or following Helicobacter pylori eradication. We aimed to evaluate the efficacy and safety of Saccharomyces boulardii in preventing antibiotic-associated diarrhoea in patients receiving antibiotics for H. pylori eradication.

METHODS: In a multicentre prospective clinical trial, patients with peptic ulcer disease or non-ulcer dyspepsia were enrolled to receive clarithromycin, amoxicillin and omeprazole for H. pylori eradication for 14 days. These patients were then randomized to receive either S. boulardii 500 mg twice daily (treatment group) or no treatment (control group). The primary outcome measure was the development of diarrhoea during (treatment period) or within 4 weeks after treatment (follow-up period).

RESULTS: Of the 389 patients that were enrolled, 376 completed the study. Within the treatment period, diarrhoea developed in 5.9% of patients in the treatment group and in 11.5% of patients in the control group (P = 0.049); and in the follow-up period, diarrhoea developed in 1.0% of patients in the treatment group and in 3.8% of patients in the control group (P = 0.09). Overall diarrhoea rates throughout the whole study period were 6.9% in the treatment group and 15.6% in the control group (P = 0.007). No significant difference was observed between the treatment and control groups in terms of adverse events.

CONCLUSION: S. boulardii is an effective and safe treatment for prevention of antibiotic-associated diarrhoea when given concomitantly to patients receiving H. pylori eradication.

L7 ANSWER 65 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2005:318613 BIOSIS Full-text
DN PREV200510103423
TI Nutritional and toxicological evaluation of Saccharomyces cerevisae fermented cassava flour.
AU Oboh, G. [Reprint Author]; Akindahunsi, A. A.
CS Fed Univ Technol, Dept Biochem, PMB 704, Akure, Nigeria goboh2001@yahoo.com
SO Journal of Food Composition and Analysis, (NOV 2005) Vol. 18, No. 7, pp. 731-738.
CODEN: JFCAEE. ISSN: 0889-1575.
DT Article
LA English
ED Entered STN: 17 Aug 2005
Last Updated on STN: 17 Aug 2005
AB Pure strain of Saccharomyces cerevisae was used to ferment cassava pulp for 72 h with the aim of increasing the protein content of the cassava product. The mash obtained was processed to cassava flour, one of the forms in which cassava product is commonly consumed in Nigeria. The nutritional and toxicological potentials of the fungus fermented cassava flour were evaluated using rat bioassay. S. cerevisae fermented cassava flour (40%) fed to albino rat for 21 days had high feed conversion and digestibility (apparent and dry matter). Moreover, this level of cassava incorporation had no negative haematological (packed cell volume, red blood cell counts and white blood cell counts) effect. However, there was a significant ($P < 0.05$) rise in the serum glutamate pyruvate transaminase and serum glutamate oxaloacetate transaminase activities indicating a possible damage to the liver (hepatotoxic) and/or heart (cardiotoxic), while there was no significant ($P > 0.05$) rise in the serum albumin and bilirubin. Further pathological investigation revealed that the spleen showed some dark red colouration while the liver had some necrotic lesion. The possible cause of this damage is the theme of further investigation in our laboratory. (c) 2004 Elsevier Inc. All rights reserved.

L7 ANSWER 66 OF 184 MEDLINE ® on STN DUPLICATE 55
AN 2005442724 MEDLINE Full-text
DN PubMed ID: 16108803
TI Use of RAPD and killer toxin sensitivity in Saccharomyces cerevisiae strain typing.
AU Corte L; Lattanzi M; Buzzini P; Bolano A; Fatichenti F; Cardinali G
CS Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali, Sezione Microbiologia Applicata, Universita degli Studi di Perugia, Borgo 20 Giugno 74, I-06121 Perugia, Italy.
SO Journal of applied microbiology, (2005) Vol. 99, No. 3, pp. 609-17.
Journal code: 9706280. ISSN: 1364-5072. L-ISSN: 1364-5072.
CY England: United Kingdom
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200602
ED Entered STN: 20 Aug 2005
Last Updated on STN: 8 Feb 2006
Entered Medline: 7 Feb 2006
AB AIMS: Two different strain characterization techniques, random amplified polymorphic DNA (RAPD) and killer toxin sensitivity (KTS), were compared to assess their typing performance using a set of 30 certified Saccharomyces cerevisiae strains.

METHODS AND RESULTS: A sequential random resampling procedure was employed to subdivide the 32 descriptors in eight sets, in order to compare the differential performances of the two techniques with diverse number of characters. Results showed that RAPD performs better than killer, although the complete differentiation of the strains under study could be obtained only by combining profiles from the two techniques.

CONCLUSIONS: The combination of different typing techniques was useful when discriminating similar organisms. In such cases, the introduction of a second typing technique can be more advantageous than increasing the number of characters obtained with a single method.

SIGNIFICANCE AND IMPACT OF THE STUDY: The distribution of among-strains pairwise distances and the relative performance of the two techniques has implications for the study of biodiversity, taxonomy and microbial ecology.

L7 ANSWER 67 OF 184 MEDLINE ® on STN DUPLICATE 56
AN 2005112867 MEDLINE Full-text
DN PubMed ID: 15498024
TI Manganese toxicity and Saccharomyces cerevisiae Mam3p, a member of the ACDP (ancient conserved domain protein) family.
AU Yang Mei; Jensen Laran T; Gardner Allison J; Culotta Valeria C
CS Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205, USA.
NC ES 07141 (United States NIEHS NIH HHS)
ES 08996 (United States NIEHS NIH HHS)
SO The Biochemical journal, (2005 Mar 15) Vol. 386, No. Pt 3, pp. 479-87.
Journal code: 2984726R. E-ISSN: 1470-8728. L-ISSN: 0264-6021.
Report No.: NLM-PMC1134866.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200508
ED Entered STN: 4 Mar 2005
Last Updated on STN: 26 Aug 2005
Entered Medline: 25 Aug 2005
OSC.G 6 There are 6 MEDLINE records that cite this record
REM.CNT 48 There are 48 cited references available in MEDLINE for this document.
AB Manganese is an essential, but potentially toxic, trace metal in biological systems. Overexposure to manganese is known to cause neurological deficits in humans, but the pathways that lead to manganese toxicity are largely unknown. We have employed the bakers' yeast Saccharomyces cerevisiae as a model system to identify genes that contribute to manganese-related damage. In a genetic screen for yeast manganese-resistance mutants, we identified S. cerevisiae MAM3 as a gene which, when deleted, would increase cellular tolerance to toxic levels of manganese and also increased the cell's resistance towards cobalt and zinc. By sequence analysis, Mam3p shares strong similarity with the mammalian ACDP (ancient conserved domain protein) family of polypeptides. Mutations in human ACDP1 have been associated with urofacial (Ochoa) syndrome. However, the functions of eukaryotic ACDPs remain unknown. We show here that S. cerevisiae MAM3 encodes an integral membrane protein of the yeast vacuole whose expression levels directly correlate with the degree of manganese toxicity. Surprisingly, Mam3p contributes to manganese toxicity without any obvious changes in vacuolar accumulation of metals. Furthermore, through genetic epistasis studies, we demonstrate that MAM3 operates independently of the well-established manganese-trafficking pathways in yeast, involving the manganese transporters Pmr1p, Smf2p and Pho84p. This is the first report of a eukaryotic ACDP family protein involved in metal homoeostasis.

L7 ANSWER 68 OF 184 MEDLINE ® on STN DUPLICATE 57
AN 2005461288 MEDLINE Full-text
DN PubMed ID: 16128673
TI Meta-analysis: non-pathogenic yeast Saccharomyces boulardii in the prevention of antibiotic-associated diarrhoea.
AU Szajewska H; Mrukowicz J
CS Department of Pediatric Gastroenterology and Nutrition, The Medical University of Warsaw, Warsaw, Poland. hania@ipgate.pl
SO Alimentary pharmacology & therapeutics, (2005 Sep 1) Vol. 22, No. 5, pp. 365-72. Ref: 39
Journal code: 8707234. ISSN: 0269-2813. L-ISSN: 0269-2813.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(META-ANALYSIS)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
General Review; (REVIEW)
LA English
FS MEDLINE; Priority Journals
EM 200511
ED Entered STN: 31 Aug 2005
Last Updated on STN: 15 Nov 2005
Entered Medline: 14 Nov 2005
OSC.G 4 There are 4 MEDLINE records that cite this record
RE.CNT 39 There are 39 cited references for this document.
AB BACKGROUND: Antibiotic-associated diarrhoea occurs in up to 30% of patients who receive antibiotics but can be prevented with probiotics.

AIM: To systematically evaluate the effectiveness of Saccharomyces boulardii in preventing antibiotic-associated diarrhoea in children and adults.

METHODS: Using medical subject headings and free-language terms, the following electronic databases were searched for studies relevant to antibiotic-associated diarrhoea and S. boulardii: MEDLINE, EMBASE, CINAHL and The Cochrane Library. Additional sources were obtained from references in reviewed articles. Only randomized-controlled trials were considered for study inclusion.

RESULTS: Of 16 potentially relevant clinical trials identified, five randomized-controlled trials (1076 participants) met the inclusion criteria for this systematic review. Treatment with S. boulardii compared with placebo reduced the risk of antibiotic-associated diarrhoea from 17.2% to 6.7% (RR: 0.43; 95% CI: 0.23-0.78; random effect model). The number needed to treat to prevent one case of antibiotic-associated diarrhoea was 10 (95% CI: 7-16). No side-effects were reported.

CONCLUSIONS: A meta-analysis of data from five randomized-controlled trials showed that S. boulardii is moderately effective in preventing antibiotic-associated diarrhoea in children and adults treated with antibiotics for

any reason (mainly respiratory tract infections). For every 10 patients receiving daily S. boulardii with antibiotics, one fewer will develop antibiotic-associated diarrhoea.

L7 ANSWER 69 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 58
AN 2005:339808 BIOSIS Full-text
DN PREV200510123798
TI Production and effect of killer toxin by Saccharomyces cerevisiae and Pichia kluyveri on sensitive yeasts and fungal pathogens.
AU Dabhole, Madhusudan P.; Joishy, K. N. [Reprint Author]
CS Univ Bombay, KJ Somaiya Coll Sci, Dept Microbiol, Bombay 400077, Maharashtra, India
drknjoishy@yahoo.co.in
SO Indian Journal of Biotechnology, (APR 2005) Vol. 4, No. 2, pp. 290-292.
ISSN: 0972-5849.
DT Article
LA English
ED Entered STN: 31 Aug 2005
Last Updated on STN: 31 Aug 2005
AB Killer yeasts from flowers of Indian medicinal plants were isolated and the effect of their killer toxin was determined on sensitive yeast cells as well as fungal pathogens. The toxin of Saccharomyces cerevisiae and Pichia kluyveri inhibited Dekkera anomala accumulating methylene blue cells on Yeast Extract Peptone Dextrose agar (pH 4.2) at 21 degrees C. There was no inhibition of growth or competition between the yeast cells in the mixed population of S. cerevisiae isolated from Acalypha indica. S. cerevisiae and P. kluyveri were found to tolerate 50% and 40% glucose, while D. anomala tolerated 40% glucose. Both S. cerevisiae and P. kluyveri did not inhibit the growth of Aspergillus niger, Candida albicans and Fusarium spp.

L7 ANSWER 70 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2005:399147 BIOSIS Full-text
DN PREV200510183119
TI Expression of cholera toxin B subunit in Saccharomyces cerevisiae.
AU Arzanlou, Mohsen; Rezaee, Abbas [Reprint Author]; Shahrokhi, Nader; Hossini, Ahmad Zavaran; Yasuda, Yoko; Tochikubo, Kunio; Ahangarzadeh Rezaee, Mohammad
CS 106,Immam Zaman Alley,95 Sq, Tehran 16456, Iran
abbasrezaee@yahoo.com
SO Annals of Microbiology, (2005) Vol. 55, No. 2, pp. 145-150.
ISSN: 1590-4261.
DT Article
LA English
ED Entered STN: 5 Oct 2005
Last Updated on STN: 5 Oct 2005
AB Cholera toxin, secreted by Vibrio cholerae, consists of A and B subunits. Cholera toxin B subunit (CTB) is used in many scientific researches. It has already been expressed in several bacterial and plant systems. In order to express CTB protein in Saccharomyces cerevisiae, the expression plasmid pCTB83 was constructed by inserting ctxB gene in pYES2 shuttle vector. The new construct was transferred into S. cerevisiae cells and the ctxB gene was induced with 2% galactose. SDS-PAGE analysis showed the presence of CTB in yeast lysate and immunoblotting analysis of yeast total soluble protein indicated that the yeast-derived CTB protein was antigenically indistinguishable from bacterial CTB protein. Quantitative ELISA showed that the maximum amount of CTB protein expressed in yeast was approximately 1.8% of total soluble protein. CTB is a subunit vaccine candidate against cholera. Since the whole recombinant yeast has been introduced as a new vaccine formulation, expression of ctxB in S. cerevisiae may offer an effective and inexpensive strategy to protect people against cholera in high-risk areas.

L7 ANSWER 71 OF 184 MEDLINE ® on STN DUPLICATE 59
AN 2005240383 MEDLINE Full-text
DN PubMed ID: 15641941
TI Cellular factors required for protection from hyperoxia toxicity in Saccharomyces cerevisiae.
AU Outten Caryn E; Falk Robert L; Culotta Valeria C
CS Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe St. Room 7032, Baltimore, MD 21205, USA.
NC ES 07141 (United States NIEHS NIH HHS)
F32 GM066594 (United States NIGMS NIH HHS)
GM 50016 (United States NIGMS NIH HHS)
GM 66594 (United States NIGMS NIH HHS)
SO The Biochemical journal, (2005 May 15) Vol. 388, No. Pt 1, pp. 93-101.
Journal code: 2984726R. E-ISSN: 1470-8728. L-ISSN: 0264-6021.
Report No.: NLM-PMC1186697.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, N.I.H., EXTRAMURAL)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200909
ED Entered STN: 10 May 2005
Last Updated on STN: 14 Dec 2005
Entered Medline: 25 Sep 2009
OSC.G 4 There are 4 MEDLINE records that cite this record
REM.CNT 46 There are 46 cited references available in MEDLINE for this document.
AB Prolonged exposure to hyperoxia represents a serious danger to cells, yet little is known about the specific cellular factors that affect hyperoxia stress. By screening the yeast deletion library, we have identified genes that protect against high-O2 damage. Out of approx. 4800 mutants, 84 were identified as hyperoxia-sensitive, representing genes with diverse cellular functions, including transcription and translation, vacuole function, NADPH production, and superoxide detoxification. Superoxide plays a significant role, since the majority of hyperoxia-sensitive mutants displayed cross-sensitivity to superoxide-generating agents, and mutants with compromised SOD (superoxide dismutase) activity were particularly vulnerable to hyperoxia. By comparison, factors known to guard against H2O2 toxicity were poorly represented amongst hyperoxia-sensitive mutants. Although many cellular components are potential targets, our

studies indicate that mitochondrial glutathione is particularly vulnerable to hyperoxia damage. During hyperoxia stress, mitochondrial glutathione is more susceptible to oxidation than cytosolic glutathione. Furthermore, two factors that help maintain mitochondrial GSH in the reduced form, namely the NADH kinase Pos5p and the mitochondrial glutathione reductase (Glr1p), are critical for hyperoxia resistance, whereas their cytosolic counterparts are not. Our findings are consistent with a model in which hyperoxia toxicity is manifested by superoxide-related damage and changes in the mitochondrial redox state.

L7 ANSWER 72 OF 184 MEDLINE ® on STN DUPLICATE 60
AN 2005025226 MEDLINE Full-text
DN PubMed ID: 15608068
TI Global network analysis of phenotypic effects: protein networks and toxicity modulation in Saccharomyces cerevisiae.
AU Said Maya R; Begley Thomas J; Oppenheim Alan V; Lauffenburger Douglas A; Samson Leona D
CS Digital Signal Processing Group, Department of Electrical Engineering and Computer Science, and Biological Engineering Division and Center for Environmental Health Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.
NC F32-ES11733 (United States NIEHS NIH HHS)
P30-ES02109 (United States NIEHS NIH HHS)
R01-CA-55042 (United States NCI NIH HHS)
U19-ES11399 (United States NIEHS NIH HHS)
SO Proceedings of the National Academy of Sciences of the United States of America, (2004 Dec 28) Vol. 101, No. 52, pp. 18006-11. Electronic Publication Date: 17 Dec 2004
Journal code: 7505876. ISSN: 0027-8424. L-ISSN: 0027-8424.
Report No.: NLM-PMC539745.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200502
ED Entered STN: 19 Jan 2005
Last Updated on STN: 1 Mar 2005
Entered Medline: 28 Feb 2005
OSC.G 27 There are 27 MEDLINE records that cite this record
REM.CNT 30 There are 30 cited references available in MEDLINE for this document.
AB Using genome-wide information to understand holistically how cells function is a major challenge of the postgenomic era. Recent efforts to understand molecular pathway operation from a global perspective have lacked experimental data on phenotypic context, so insights concerning biologically relevant network characteristics of key genes or proteins have remained largely speculative. Here, we present a global network investigation of the genotype/phenotype data set we developed for the recovery of the yeast Saccharomyces cerevisiae from exposure to DNA-damaging agents, enabling explicit study of how protein-protein interaction network characteristics may be associated with phenotypic functional effects. We show that toxicity-modulating proteins have similar topological properties as essential proteins, suggesting that cells initiate highly coordinated responses to damage similar to those needed for vital cellular functions. We also identify toxicologically important protein complexes, pathways, and modules. These results have potential implications for understanding toxicity-modulating processes relevant to a number of human diseases, including cancer and aging.

L7 ANSWER 73 OF 184 MEDLINE ® on STN DUPLICATE 61
AN 2004254781 MEDLINE Full-text
DN PubMed ID: 15153117
TI Modeling the Qo site of crop pathogens in Saccharomyces cerevisiae cytochrome b.
AU Fisher Nicholas; Brown Amanda C; Sexton Graham; Cook Alison; Windass John; Meunier Brigitte
CS The Wolfson Institute for Biomedical Research, University College London, Gower Street, London WC1E 6BT, UK.
SO European journal of biochemistry / FEBS, (2004 Jun) Vol. 271, No. 11, pp. 2264-71.
Journal code: 0107600. ISSN: 0014-2956. L-ISSN: 0014-2956.
CY Germany: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200407
ED Entered STN: 22 May 2004
Last Updated on STN: 7 Jul 2004
Entered Medline: 6 Jul 2004
OSC.G 4 There are 4 MEDLINE records that cite this record
AB Saccharomyces cerevisiae has been used as a model system to characterize the effect of cytochrome b mutations found in fungal and oomycete plant pathogens resistant to Q(o) inhibitors (QoIs), including the strobilurins, now widely employed in agriculture to control such diseases. Specific residues in the Q(o) site of yeast cytochrome b were modified to obtain four new forms mimicking the Q(o) binding site of Erysiphe graminis, Venturia inaequalis, Sphaerotheca fuliginea and Phytophthora megasperma. These modified versions of cytochrome b were then used to study the impact of the introduction of the G143A mutation on bc(1) complex activity. In addition, the effects of two other mutations F129L and L275F, which also confer levels of QoI insensitivity, were also studied. The G143A mutation caused a high level of resistance to QoI compounds such as myxothiazol, axoxystrobin and pyraclostrobin, but not to stigmatellin. The pattern of resistance conferred by F129L and L275F was different. Interestingly G143A had a slightly deleterious effect on the bc(1) function in V. inaequalis, S. fuliginea and P. megasperma Q(o) site mimics but not in that for E. graminis. Thus small variations in the Q(o) site seem to affect the impact of the G143A mutation on bc(1) activity. Based on this observation in the yeast model, it might be anticipated that the G143A mutation might affect the fitness of pathogens differentially. If so, this could contribute to observed differences in the rates of evolution of QoI resistance in fungal and oomycete pathogens.

L7 ANSWER 74 OF 184 MEDLINE ® on STN DUPLICATE 62
AN 2004501185 MEDLINE Full-text

DN PubMed ID: 15470257
TI FKBP12 controls aspartate pathway flux in Saccharomyces cerevisiae to prevent toxic intermediate accumulation.
AU Arevalo-Rodriguez Miguel; Pan Xuewen; Boeke Jef D; Heitman Joseph
CS Department of Molecular Genetics and Microbiology, Box 3546, 322 CARL Building, Research Dr., Duke University Medical Center, Durham, NC 27710, USA.
NC R01 AI50438 (United States NIAID NIH HHS)
R01 HG002432 (United States NHGRI NIH HHS)
SO Eukaryotic cell, (2004 Oct) Vol. 3, No. 5, pp. 1287-96.
Journal code: 101130731. ISSN: 1535-9778. L-ISSN: 1535-9786.
Report No.: NLM-PMC522611.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200503
ED Entered STN: 8 Oct 2004
Last Updated on STN: 12 Mar 2005
Entered Medline: 11 Mar 2005
OSC.G 5 There are 5 MEDLINE records that cite this record
REM.CNT 78 There are 78 cited references available in MEDLINE for this document.

AB FKBP12 is a conserved member of the prolyl-isomerase enzyme family and serves as the intracellular receptor for FK506 that mediates immunosuppression in mammals and antimicrobial actions in fungi. To investigate the cellular functions of FKBP12 in Saccharomyces cerevisiae, we employed a high-throughput assay to identify mutations that are synthetically lethal with a mutation in the FPR1 gene, which encodes FKBP12. This screen identified a mutation in the HOM6 gene, which encodes homoserine dehydrogenase, the enzyme catalyzing the last step in conversion of aspartic acid into homoserine, the common precursor in threonine and methionine synthesis. Lethality of fpr1 hom6 double mutants was suppressed by null mutations in HOM3 or HOM2, encoding aspartokinase and aspartate beta-semialdehyde dehydrogenase, respectively, supporting the hypothesis that fpr1 hom6 double mutants are inviable because of toxic accumulation of aspartate beta-semialdehyde, the substrate of homoserine dehydrogenase. Our findings also indicate that mutation or inhibition of FKBP12 dysregulates the homoserine synthetic pathway by perturbing aspartokinase feedback inhibition by threonine. Because this pathway is conserved in fungi but not in mammals, our findings suggest a facile route to synergistic antifungal drug development via concomitant inhibition of FKBP12 and Hom6.

L7 ANSWER 75 OF 184 MEDLINE ® on STN DUPLICATE 63
AN 2004164456 MEDLINE Full-text
DN PubMed ID: 15047528
TI Chemosensitization of fluconazole resistance in Saccharomyces cerevisiae and pathogenic fungi by a D-octapeptide derivative.
AU Niimi K; Harding D R K; Parshot R; King A; Lun D J; Decottignies A; Niimi M; Lin S; Cannon R D; Goffeau A; Monk B C
CS Department of Oral Sciences, University of Otago, Dunedin, New Zealand.
SO Antimicrobial agents and chemotherapy, (2004 Apr) Vol. 48, No. 4, pp. 1256-71.
Journal code: 0315061. ISSN: 0066-4804. L-ISSN: 0066-4804.
Report No.: NLM-PMC375246.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200405
ED Entered STN: 3 Apr 2004
Last Updated on STN: 25 May 2004
Entered Medline: 24 May 2004
OSC.G 11 There are 11 MEDLINE records that cite this record
REM.CNT 50 There are 50 cited references available in MEDLINE for this document.

AB Hyperexpression of the Saccharomyces cerevisiae multidrug ATP-binding cassette (ABC) transporter Pdr5p was driven by the pdr1-3 mutation in the Pdr1p transcriptional regulator in a strain (AD/PDR5(+)) with deletions of five other ABC-type multidrug efflux pumps. The strain had high-level fluconazole (FLC) resistance (MIC, 600 microg ml(-1)), and plasma membrane fractions showed oligomycin-sensitive ATPase activity up to fivefold higher than that shown by fractions from an isogenic PDR5-null mutant (FLC MIC, 0.94 microg ml(-1)). In vitro inhibition of the Pdr5p ATPase activity and chemosensitization of cells to FLC allowed the systematic screening of a 1.8-million-member designer D-octapeptide combinatorial library for surface-active Pdr5p antagonists with modest toxicity against yeast cells. Library deconvolution identified the 4-methoxy-2,3,6-trimethylbenzensulfonyl-substituted D-octapeptide KN20 as a potent Pdr5p ATPase inhibitor (concentration of drug causing 50% inhibition of enzyme activity [IC(50)], 4 microM) which chemosensitized AD/PDR5(+) to FLC, itraconazole, and ketoconazole. It also inhibited the ATPase activity of other ABC transporters, such as Candida albicans Cdr1p (IC(50), 30 microM) and Cdr2p (IC(50), 2 microM), and chemosensitized clinical isolates of pathogenic Candida species and S. cerevisiae strains that heterologously hyperexpressed either ABC-type multidrug efflux pumps, the C. albicans major facilitator superfamily-type drug transporter Ben(R)p, or the FLC drug target lanosterol 14 alpha-demethylase (Erg11p). Although KN20 also inhibited the S. cerevisiae plasma membrane proton pump Pma1p (IC(50), 1 microM), the peptide concentrations required for chemosensitization made yeast cells permeable to rhodamine 6G. KN20 therefore appears to indirectly chemosensitize cells to FLC by a nonlethal permeabilization of the fungal plasma membrane.

L7 ANSWER 76 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2006:159134 BIOSIS Full-text
DN PREV200600154594
TI Astonishing mutant gene enabling the conversion of cellulosic biomass to ethanol fuel and green chemicals by the safe effective saccharomyces yeast.
AU Ho, Nancy W. Y. [Reprint Author]
CS Purdue Univ, Renewable Resources Engn Lab LORRE, Potter Engn Ctr, W Lafayette, IN 47907 USA
nwyho@ecn.purdue.edu
SO Abstracts of Papers American Chemical Society, (MAR 28 2004) Vol. 227, No. Part 1, pp. U299.

Meeting Info.: 227th National Meeting of the American-Chemical Society. Anaheim, CA, USA. March 28 -April 01, 2004. Amer Chem Soc.
CODEN: ACSRAL. ISSN: 0065-7727.
DT Conference; (Meeting)
Conference; Abstract; (Meeting Abstract)
LA English
ED Entered STN: 9 Mar 2006
Last Updated on STN: 9 Mar 2006

L7 ANSWER 77 OF 184 MEDLINE ® on STN DUPLICATE 64
AN 2004131969 MEDLINE Full-text
DN PubMed ID: 14735355
TI Analyses of the effects of Rck2p mutants on Pbs2pDD-induced toxicity in Saccharomyces cerevisiae identify a MAP kinase docking motif, and unexpected functional inactivation due to acidic substitution of T379.
AU Jiang L; Niu S; Clines K L; Burke D J; Sturgill T W
CS Department of Pharmacology, University of Virginia Health Sciences Center, PO Box 800735, Charlottesville, VA 22908, USA.
NC GM62890 (United States NIGMS NIH HHS)
SO Molecular genetics and genomics : MGG, (2004 Mar) Vol. 271, No. 2, pp. 208-19. Electronic Publication Date: 21 Jan 2004
Journal code: 101093320. ISSN: 1617-4615. L-ISSN: 1617-4623.
CY Germany: Germany, Federal Republic of
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200405
ED Entered STN: 17 Mar 2004
Last Updated on STN: 10 May 2004
Entered Medline: 7 May 2004
OSC.G 2 There are 2 MEDLINE records that cite this record
AB Rck2p is a Ser/Thr kinase that binds to, and is activated by, Hog1p. Expression of the MAP kinase kinase Pbs2pDD from a GAL1-driven plasmid hyperactivates the HOG MAP kinase pathway, and leads to cessation of growth. This toxic effect is reduced by deletion of RCK2. We studied the structural and functional basis for the role of Rck2p in mediating the growth arrest phenotype associated with overexpression of Pbs2pDD. Rck2p kinase activity is required for the effect, because Rck2p(Delta487-610), as well as full-length Rck2p, is toxic with Pbs2pDD, but kinase-defective versions of either protein with a K201R mutation are not. Thus, the C-terminal portion of Rck2p is not required provided the protein is activated by removal of the autoinhibitory domain. Relief of inhibition in Rck2p normally requires phosphorylation by Hog1p, and Rck2p contains a putative MAP kinase docking site (TILQR589R590KKVQ) in its C-terminal segment. The Rck2p double mutant R589A/R590A expressed from a centromeric plasmid did not detectably bind Hog1p-GFP and was functionally inactive in mediating the toxic effect of Pbs2pDD, equivalent to an RCK2 deletion. However, overexpression of Rck2p R589A/R590A from a multicopy plasmid restored function. In contrast, RCK2-K201R acted as a multicopy suppressor of PBS2DD, markedly reducing its toxicity. This suppressor activity required the K201R mutation, and the effect was largely lost when the docking site was mutated, suggesting suppression by inhibition of Hog1p functions. We also studied the effect of replacing the predicted T379 and established S520 phosphorylation sites in Rck2p by glutamic acid. Surprisingly, the T379E mutant markedly reduced Pbs2pDD toxicity, and toxicity was only partially rescued by S520E. Rck2 T379E was sufficiently inactive in an rck2Delta strain to allow some cells to survive PBS2DD toxicity even when overexpressed. The significance of these findings for our understanding of Rck2p function is discussed.

L7 ANSWER 78 OF 184 MEDLINE ® on STN DUPLICATE 65
AN 2004393952 MEDLINE Full-text
DN PubMed ID: 15297032
TI An examination of quinone toxicity using the yeast Saccharomyces cerevisiae model system.
AU Rodriguez Chester E; Shinyashiki Masaru; Froines John; Yu Rong Chun; Fukuto Jon M; Cho Arthur K
CS Department of Pharmacology, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, CA 90095-1735, USA.
SO Toxicology, (2004 Sep 1) Vol. 201, No. 1-3, pp. 185-96.
Journal code: 0361055. ISSN: 0300-483X. L-ISSN: 0300-483X.
CY Ireland
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200410
ED Entered STN: 7 Aug 2004
Last Updated on STN: 13 Oct 2004
Entered Medline: 12 Oct 2004
OSC.G 2 There are 2 MEDLINE records that cite this record
AB The toxicity of quinones is generally thought to occur by two mechanisms: the formation of covalent bonds with biological molecules by Michael addition chemistry and the catalytic reduction of oxygen to superoxide and other reactive oxygen species (ROS) (redox cycling). In an effort to distinguish between these general mechanisms of toxicity, we have examined the toxicity of five quinones to yeast cells as measured by their ability to reduce growth rate. Yeast cells can grow in the presence and absence of oxygen and this feature was used to evaluate the role of redox cycling in the toxicity of each quinone. Furthermore, yeast mutants deficient in superoxide dismutase (SOD) activity were used to assess the role of this antioxidant enzyme in protecting cells against quinone-induced reactive oxygen toxicity. The effects of different quinones under different conditions of exposure were compared using IC50 values (the concentration of quinone required to inhibit growth rate by 50%). For the most part, the results are consistent with the chemical properties of each quinone with the exception of 9,10-phenanthrenequinone (9,10-PQ). This quinone, which is not an electrophile, exhibited an unexpected toxicity under anaerobic conditions. Further examination revealed a potent induction of cell viability loss which poorly correlated with decreases in the GSH/2GSSG ratio but highly correlated (r2 > 0.7) with inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), suggesting disruption of glycolysis by this quinone. Together, these observations suggest an unexpected oxygen-independent mechanism in the toxicity of 9,10-phenanthrenequinone.

L7 ANSWER 79 OF 184 MEDLINE ® on STN DUPLICATE 66
AN 2003579803 MEDLINE Full-text
DN PubMed ID: 12954629

TI Frelp Cu2+ reduction and Fet3p Cu1+ oxidation modulate copper toxicity in Saccharomyces cerevisiae.
AU Shi Xiaoli; Stoj Christopher; Romeo Annette; Kosman Daniel J; Zhu Zhiwu
CS Department of Environmental Toxicology, University of California, Santa Cruz, California 95064, USA.
NC DK53820 (United States NIDDK NIH HHS)
SO The Journal of biological chemistry, (2003 Dec 12) Vol. 278, No. 50, pp. 50309-15. Electronic Publication Date: 3 Sep 2003
Journal code: 2985121R. ISSN: 0021-9258. L-ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200401
ED Entered STN: 16 Dec 2003
Last Updated on STN: 16 Jan 2004
Entered Medline: 15 Jan 2004
OSC.G 8 There are 8 MEDLINE records that cite this record
AB Frelp is a metalloreductase in the yeast plasma membrane that is essential to uptake of environmental Cu2+ and Fe3+. Fet3p is a multicopper oxidase in this membrane essential for high affinity iron uptake. In the uptake of Fe3+, Frelp produces Fe2+ that is a substrate for Fet3p; the Fe3+ produced by Fet3p is a ligand for the iron permease, Ftrlp. Deletion of FET3 leads to iron deficiency; this deletion also causes a copper sensitivity not seen in wild type. Deletion of FTR1 leads to copper sensitivity also. Production in the ftr1delta strain of an iron-uptake negative Ftrlp mutant, Ftrlp(RAGLA), suppressed this copper sensitivity. This Ftrlp mutant supported the plasma membrane targeting of active Fet3p that is blocked in the parental ftr1delta strain. A ferroxidase-negative Fet3p did not suppress the copper sensitivity in a fet3delta strain, although it supported the plasma membrane localization of the Fet3p.Ftrlp complex. Thus, loss of membrane-associated Fet3p oxidase activity correlated with copper sensitivity. Furthermore, in vitro Cu1+ was shown to be an excellent substrate for Fet3p. Last, the copper sensitivity of the fet3delta strain was suppressed by co-deletion of FRE1, suggesting that the cytotoxic species was Cu1+. In contrast, deletion of CTR1 or of FET4 did not suppress the copper sensitivity in the fet3delta strain; these genes encode the two major copper transporters in laboratory yeast strains. This result indicated that the apparent cuprous ion toxicity was not due to excess intracellular copper. These biochemical and physiologic results indicate that at least with respect to cuprous and ferrous ions, Fet3p can be considered a metallo-oxidase and appears to play an essential role in both iron and copper homeostasis in yeast. Its functional homologs, e.g. ceruloplasmin and hephaestin, could play a similar role in mammals.

L7 ANSWER 80 OF 184 MEDLINE ® on STN DUPLICATE 67
AN 2004006872 MEDLINE Full-text
DN PubMed ID: 14703947
TI Effect of expression of the Escherichia coli nth gene in Saccharomyces cerevisiae on the toxicity of ionizing radiation and hydrogen peroxide.
AU Skorvaga M; Cernakova L; Chovanec M; Vlasakova D; Kleibl K; Hendry J H; Margison G P; Brozmanova J
CS Laboratory of Molecular Genetics, Cancer Research Institute, Slovak Academy of Sciences, Vlarska 7, 833 91 Bratislava 37, Slovak Republic.
SO International journal of radiation biology, (2003 Sep) Vol. 79, No. 9, pp. 747-55.
Journal code: 8809243. ISSN: 0955-3002. L-ISSN: 0955-3002.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals; Space Life Sciences
EM 200403
ED Entered STN: 6 Jan 2004
Last Updated on STN: 6 Mar 2004
Entered Medline: 5 Mar 2004
AB PURPOSE: To examine the contribution of endonuclease III (Nth)-repairable lesions to the cytotoxicity of ionizing radiation (IR) and hydrogen peroxide (H2O2) in the yeast Saccharomyces cerevisiae.

MATERIALS AND METHODS: A selectable expression vector containing the E. coli nth gene was transformed into two different wild-type strains (7799-4B and YNN-27) as well as one rad52 mutant strain (C5-6). Nth expression was verified by Western analysis. Colony-forming assay was used to determine the sensitivity to IR and H2O2 in both stationary and exponentially growing cells.

RESULTS: The pADHnth-transformed wild-type (77994B) strain was considerably more resistant than vector-only transformants to the toxic effects of IR, in both stationary and exponential growth phases, although this was not the case in another wild-type strain (YNN-27). In contrast, there were no significant effects of nth expression on the sensitivity of the wild-type cells to H2O2. Moreover, nth expression caused no effects on the H2O2 sensitivity in the rad52 mutant cells, but it led to a slight increase in sensitivity in these cells following IR, particularly at the highest dose levels used.

CONCLUSIONS: Whilst other damage-processing systems may play a role, DNA lesions that are substrates for Nth can also make a contribution to the toxic effects of IR in certain wild-type yeast. Hence, DNA double-strand breaks should not be considered the sole lethal lesions following IR exposure.

L7 ANSWER 81 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2003:428668 BIOSIS Full-text
DN PREV200300428668
TI Resistance of Saccharomyces cerevisiae to toxic lignocellulose-derived aromatic compounds.
AU Bjorklund, Linda [Reprint Author]; Larsson, Simona; Martin, Carlos; Horvath, Ilona Sarvari; Alriksson, Bjorn; Jonsson, Leif J.
CS AstraZeneca R and D Molndal, SE-431 83, Molndal, Sweden
Leif.Jonsson@kau.se
SO Yeast, (July 2003) Vol. 20, No. Supplement 1, pp. S206. print.
Meeting Info.: XXIst International Conference on Yeast Genetics and

Molecular Biology. Goeteborg, Sweden. July 07-12, 2003.
ISSN: 0749-503X (ISSN print).
DT Conference; (Meeting)
Conference; Abstract; (Meeting Abstract)
LA English
ED Entered STN: 17 Sep 2003
Last Updated on STN: 17 Sep 2003

L7 ANSWER 82 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2003:417572 BIOSIS Full-text
DN PREV200300417572
TI Functional characterization of oxysterol-binding proteins in budding yeast Saccharomyces cerevisiae and pathogenic yeast Candida albicans.
AU Ryu, Ji-ho [Reprint Author]; Kim, Kwang-hoon [Reprint Author]; Huh, Hyangsuk [Reprint Author]; Kim, Jinmi [Reprint Author]
CS Microbiology, Chungnam National University, KungDong 220, Taejeon, 305-764, South Korea
jmkim@cnu.ac.kr
SO Yeast, (July 2003) Vol. 20, No. Supplement 1, pp. S77. print.
Meeting Info.: XXIst International Conference on Yeast Genetics and Molecular Biology. Goeteborg, Sweden. July 07-12, 2003.
ISSN: 0749-503X (ISSN print).
DT Conference; (Meeting)
Conference; (Meeting Poster)
Conference; Abstract; (Meeting Abstract)
LA English
ED Entered STN: 10 Sep 2003
Last Updated on STN: 10 Sep 2003

L7 ANSWER 83 OF 184 MEDLINE ® on STN DUPLICATE 68
AN 2002453114 MEDLINE Full-text
DN PubMed ID: 12208740
TI dUTPase and uracil-DNA glycosylase are central modulators of antifolate toxicity in Saccharomyces cerevisiae.
AU Tinkelenberg Beverly A; Hansbury Michael J; Ladner Robert D
CS Department of Molecular Biology, University of Medicine and Dentistry of New Jersey, School of Osteopathic Medicine, Stratford, New Jersey 08084, USA.
NC CA83861 (United States NCI NIH HHS)
SO Cancer research, (2002 Sep 1) Vol. 62, No. 17, pp. 4909-15.
Journal code: 2984705R. ISSN: 0008-5472. L-ISSN: 0008-5472.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 200210
ED Entered STN: 6 Sep 2002
Last Updated on STN: 4 Oct 2002
Entered Medline: 3 Oct 2002
OSC.G 12 There are 12 MEDLINE records that cite this record
AB The thymidylate synthase reaction remains an important target for widely used anticancer agents; however, the clinical utility of these drugs is limited by the occurrence of cellular resistance. Despite the considerable amount of information available regarding mechanisms of drug action, the relative significance of downstream events that result in lethality remains unclear. In this study, we have developed a model system using the budding yeast Saccharomyces cerevisiae to dissect the influence of dUMP misincorporation into DNA as a contributing mechanism of cytotoxicity induced by antifolate agents. The activities of dUTPase and uracil-DNA glycosylase, key enzymes in uracil-DNA metabolism, were diminished or augmented, and the manipulated strains were analyzed for biochemical endpoints of toxicity. Cells overexpressing dUTPase were protected from cytotoxicity by their ability to prevent dUTP pool expansion and were able to recover from an early S-phase checkpoint arrest. In contrast, depletion of dUTPase activity leads to the accumulation of dUTP pools and enhanced sensitivity to antifolates. These cells were also arrested in early S-phase and were unable to complete DNA replication after drug withdrawal, resulting in lethality. Inactivation of uracil base excision repair induced partial resistance to early cytotoxicity (within 10 h); however, lethality ultimately resulted at later time points (12-24 h), presumably because of the detrimental effects of stable uracil misincorporation. Although these cells were able to complete replication with uracil-substituted DNA, they arrested at the G(2)-M phase. This finding may represent a novel mechanism by which the G(2)-M checkpoint is signaled by the presence of uracil-substituted DNA. Together these data provide both genetic and biochemical evidence demonstrating that lethality from antifolates in yeast is primarily dependent on uracil misincorporation into DNA, and that uracil-independent mechanisms associated with dTTP depletion play a minor role. Our findings indicate that the relative expression levels of both dUTPase and uracil-DNA glycosylase can have great influence over the efficacy of thymidylate synthase-directed chemotherapy, thereby enhancing the candidacy of these proteins as prognostic markers and alternative targets for therapeutic development.

L7 ANSWER 84 OF 184 MEDLINE ® on STN DUPLICATE 69
AN 2002259283 MEDLINE Full-text
DN PubMed ID: 11999768
TI Structure-activity relationships and response-surface analysis of nitroaromatics toxicity to the yeast (Saccharomyces cerevisiae).
AU Wang Xiaodong; Yin Chunsheng; Wang Liansheng
CS State Key Laboratory of Pollution Control and Resources Reuse, The School of Environment, Nanjing University, China.
SO Chemosphere, (2002 Feb) Vol. 46, No. 7, pp. 1045-51.
Journal code: 0320657. ISSN: 0045-6535. L-ISSN: 0045-6535.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200212
ED Entered STN: 10 May 2002
Last Updated on STN: 27 Dec 2002
Entered Medline: 26 Dec 2002

OSC.G 1 There are 1 MEDLINE records that cite this record
AB Inhibition of growth of the yeast Saccharomyces cerevisiae (Cmiz, the minimum concentration that produced a clear inhibition zone within 12 h) for 24 nitroaromatic compounds was investigated and a quantitative structure-activity relationship (QSAR) developed based on hydrophobicity expressed as the 1-octanol/water partition coefficient in logarithm form, log K(ow), electrophilicity based on the energy of the lowest unoccupied orbital (E(lumo)). All nitrobenzene derivatives exhibited enhanced reactive toxicity than baseline. The toxicities of mono-nitrobenzenes and di-nitrobenzenes were elicited by different mechanisms of toxic action. For mono-nitro-derivatives, both significant log K(ow) based and strong E(lumo)-dependent relationships were observed indicating that their toxicities were affected both by the penetration process and the interaction with target sites of interaction. The toxicities of di-nitrobenzenes were greater than mono-nitrobenzenes and no log K(ow)-dependent but highly significant E(lumo)-based relationship was obtained. This suggests that toxicity of di-nitrobenzenes was highly electrophilic and involved mainly their in vivo electrophilic interaction with biomacromolecules. In an effort to model the elevated toxicity of all nitrobenzenes, a response-surface analysis was performed and this resulted in a highly predictive two-variable QSAR without reference to their exact mechanisms (Cmiz = 0.41 log K(ow) - 0.89 E(lumo) - 0.46, r2 = 0.87, Q2 = 0.86, n = 24).

L7 ANSWER 85 OF 184 MEDLINE ® on STN DUPLICATE 70
AN 2002325492 MEDLINE Full-text
DN PubMed ID: 12067833
TI Experimental effects of Saccharomyces boulardii on diarrheal pathogens.
AU Czerucka Dorota; Rampal Patrick
CS Laboratoire de gastroenterologie et nutrition, universite de Nice-Sophia-Antipolis, faculte de medecine, 28 avenue de Valombrose, 06107 Nice cedex 2, France. czerucka@unice.fr
SO Microbes and infection / Institut Pasteur, (2002 Jun) Vol. 4, No. 7, pp. 733-9. Ref: 39
Journal code: 100883508. ISSN: 1286-4579. L-ISSN: 1286-4579.
CY France
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA English
FS MEDLINE; Priority Journals
EM 200208
ED Entered STN: 18 Jun 2002
Last Updated on STN: 23 Aug 2002
Entered Medline: 22 Aug 2002
OSC.G 5 There are 5 MEDLINE records that cite this record
RE.CNT 39 There are 39 cited references for this document.
AB Saccharomyces boulardii is a selected strain of yeast that may have applications in the prevention and treatment of intestinal infections. The animal models and in vitro studies developed to elucidate the mechanisms of this protection are reviewed and discussed.

L7 ANSWER 86 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 71
AN 2001:549233 BIOSIS Full-text
DN PREV200100549233
TI Saccharomyces cerevisiae, a potential pathogen towards grapevine, Vitis vinifera.
AU Gognies, Sabine; Belarbi, Abdel [Reprint author]; Ait Barka, Essaid
CS UFR Sciences, Laboratoire de Microbiologie Generale et Moleculaire, Universite de Reims, 51687, Reims Cedex 2, France
abdel.belarbi@univ-reims.fr
SO FEMS Microbiology Ecology, (October, 2001) Vol. 37, No. 2, pp. 143-150. print.
CODEN: FMECEZ. ISSN: 0168-6496.
DT Article
LA English
ED Entered STN: 21 Nov 2001
Last Updated on STN: 25 Feb 2002
AB Stresses applied to plants by pathogens such as fungi, bacteria, and viruses are well documented. However, to our knowledge, no study has focused on the effect of yeasts on plants. In this work the relationship between the growth of yeast, Saccharomyces cerevisiae, and its action on grapevine (Vitis vinifera L.) plantlets was studied. We observed that certain strains of S. cerevisiae could penetrate into the grapevine plants, bringing about a delay in the growth, or even causing the plantlets to die. We correlated this novel parasitic behavior of these strains of S. cerevisiae with their endopolygalacturonase activities and pseudohyphae formation. This study reports that the differences in behavior between the strains of S. cerevisiae are based on the filamentous forms, but that their pectolytic activities are required to invade grapevine tissues. The invasive process of the host plant has been confirmed histologically. Such yeast-plant interactions explain how S. cerevisiae may survive on grapevine throughout the years. The details of the parasitic relationship between S. cerevisiae and V. vinifera plantlets together with these parameters are discussed.

L7 ANSWER 87 OF 184 MEDLINE ® on STN DUPLICATE 72
AN 2000245478 MEDLINE Full-text
DN PubMed ID: 10783151
TI GFAT as a target molecule of methylmercury toxicity in Saccharomyces cerevisiae.
AU Naganuma A; Miura N; Kaneko S; Mishina T; Hosoya S; Miyairi S; Furuchi T; Kuge S
CS Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan. naganuma@mail.pharm.tohoku.ac.jp
SO FASEB journal : official publication of the Federation of American Societies for Experimental Biology, (2000 May) Vol. 14, No. 7, pp. 968-72.
Journal code: 8804484. ISSN: 0892-6638. L-ISSN: 0892-6638.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 200006
ED Entered STN: 13 Jun 2000
Last Updated on STN: 13 Jun 2000
Entered Medline: 1 Jun 2000

OSC.G 1 There are 1 MEDLINE records that cite this record
AB Using a genomic library constructed from Saccharomyces cerevisiae, we have identified a gene GFA1 that confers resistance to methylmercury toxicity. GFA1 encodes L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) and catalyzes synthesis of glucosamine-6-phosphate. Transformed yeast cells expressing GFA1 demonstrated resistance to methylmercury but no resistance to p-chloromercuribenzoate, a GFAT inhibitor. The cytotoxicity of methylmercury was inhibited by loading excess glucosamine 6-phosphate into yeast. Considering that GFAT is an essential cellular enzyme, our findings suggest that GFAT is the major target molecule of methylmercury in yeasts. This report is the first to identify the target molecule of methylmercury toxicity in eukaryotic cells.

L7 ANSWER 88 OF 184 MEDLINE ® on STN DUPLICATE 73
AN 2000414557 MEDLINE Full-text
DN PubMed ID: 10844656
TI Identification of genes affecting selenite toxicity and resistance in Saccharomyces cerevisiae.
AU Pinson B; Sagot I; Daignan-Fornier B
CS Institut de Biochimie et Genetique Cellulaires, CNRS UPR9026, 1, rue Camille Saint-Saens, F-33077 Bordeaux Cedex, France.
SO Molecular microbiology, (2000 May) Vol. 36, No. 3, pp. 679-87.
Journal code: 8712028. ISSN: 0950-382X. L-ISSN: 0950-382X.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200008
ED Entered STN: 7 Sep 2000
Last Updated on STN: 7 Sep 2000
Entered Medline: 29 Aug 2000
OSC.G 8 There are 8 MEDLINE records that cite this record
AB Recent studies associating dietary selenium with reduced cancer susceptibility have aroused interest in this substance. In the millimolar range, selenite is toxic and slightly mutagenic for yeast. We show that selenite-treated yeast cells tend to arrest as large budded cells and that this arrest is abolished in a rad9 mutant that is significantly sensitive to selenite. Interestingly, a rev3 mutant affected in the error-prone repair pathway is also sensitive to selenite, whereas mutations in the other DNA repair pathways do not strongly affect resistance to selenite. We propose that selenite treatment leads to DNA damage inducing the RAD9-dependent cell cycle arrest. Selenite-induced DNA damage could be converted to mutations by the Rev3p-dependent lesion bypass system, thus allowing the cell cycle to progress. We have also investigated the selenite detoxification mechanisms and identified three genes involved in this process. In the present study, we show that lack of the cadmium glutathione-conjugate vacuolar pump Ycf1p or overexpression of the sulphite resistance membrane protein Ssu1p enhance the capacity of yeast cells to resist selenite treatment. Finally, we show that overexpression of the glutathione reductase Glr1p increases resistance to selenite, suggesting that selenite toxicity in yeast is closely linked to its oxidative capacity.

L7 ANSWER 89 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2000:139126 BIOSIS Full-text
DN PREV200000139126
TI Nutritional and toxicological evaluation of yeast (Saccharomyces cerevisiae) biomass and a yeast protein concentrate.
AU Caballero-Cordoba, Glenys M.; Sgarbieri, Valdemiro C. [Reprint author]
CS Centre of Food Chemistry and Applied Nutrition, Institute of Food Technology, AV Brasil 2880, CEP 13073-001, Campinas, SP, Brazil
SO Journal of the Science of Food and Agriculture, (Feb., 2000) Vol. 80, No. 3, pp. 341-351. print.
CODEN: JSFAAE. ISSN: 0022-5142.
DT Article
LA English
ED Entered STN: 19 Apr 2000
Last Updated on STN: 4 Jan 2002
AB Brewer's yeast was prepared by alkaline treatment for debittering, cell wall rupture and dehydration by spray drying. Yeast protein concentrate was prepared by centrifugation of the ruptured cell suspension, treatment of the supernatant with sodium perchlorate, precipitation of the protein at isoelectric pH (4.2) and neutralisation of the isoelectric protein to pH 6.5 with sodium hydroxide, prior to lyophylisation. Chemical characterisation was performed on the biomass and protein concentrate. Amino acid scores were 98.1 and 87.2% for the whole biomass and protein concentrate respectively, based on available lysine and compared with the FAO/WHO/UNU reference standard. The growth-promoting property of the yeast biomass protein was roughly 85% of casein and was significantly better than for the yeast protein concentrate. No difference in growth was found between 15 and 30% dietary protein for all three sources, ie casein, whole yeast biomass and yeast protein concentrate. When tested for subchronical toxicity at 15 and 30% protein concentration, no evidence of toxicity was found for the whole yeast biomass, compared with casein, after 45 and 90 days of feeding. Retarded growth and discrete liver steatosis were observed in rats fed the yeast protein concentrate at both dietary levels.

L7 ANSWER 90 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2000:224139 BIOSIS Full-text
DN PREV200000224139
TI Interaction of Saccharomyces cerevisiae with gold: Toxicity and accumulation.
AU Karamushka, Victor I.; Gadd, Geoffrey M. [Reprint author]
CS Department of Biological Sciences, University of Dundee, Dundee, Scotland, DD1 4HN, UK
SO Biometals, (Dec., 1999-2000) Vol. 12, No. 4, pp. 289-294. print.
ISSN: 0966-0844.
DT Article
LA English
ED Entered STN: 31 May 2000
Last Updated on STN: 5 Jan 2002
AB This paper examines the effects of ionic gold on Saccharomyces cerevisiae, as determined by long-term (growth in gold-containing media) and short-term interactions (H+ efflux activity). An increasing gold concentration inhibited growth and at <0.2 mM Au, growth was not observed. Transmission electron microscopy revealed no differences in ultrastructure but fine electron dense particles were observed in unstained preparations from gold-containing medium. After glucose addition (to 10mM) to starved suspensions of S. cerevisiae, glucose-dependent reduction of external pH occurred as the cells extruded protons. In the presence of increasing gold concentrations, the lag time before proton

extrusion did not change but the rate and duration decreased significantly with a marked influence on proton efflux rate being observed at ltoreq 10 muM. Extension of preincubation time of yeast cells in gold-containing medium resulted in a decreasing proton efflux rate and colloidal phase formation in the cell suspensions, the time between gold addition and the beginning of colloidal phase formation depending on the gold concentration used. Both Ca and Mg enhanced the inhibitory effect of gold on the yeast cells with Ca showing a stronger inhibitory effect than Mg.

L7 ANSWER 91 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 74
AN 2002:253010 BIOSIS Full-text
DN PREV200200253010
TI Bioassay for chemical toxicity using yeast Saccharomyces cerevisiae.
AU Iwahashi, H. [Reprint author]; Fujita, K. [Reprint author]; Takahashi, Y. [Reprint author]
CS National Institute of Bioscience and Human-Technology, Higashi 1-1, Tsukuba, Ibaraki, 305-8566, Japan
SO Water Science and Technology, (2000) Vol. 42, No. 7-8, pp. 269-276. print.
CODEN: WSTED4. ISSN: 0273-1223.
DT Article
LA English
ED Entered STN: 24 Apr 2002
Last Updated on STN: 24 Apr 2002
AB Multi-endpoint bioassay is a system for gathering information relative to the effects of newly synthesized chemicals and pollutants in the environment. To develop the multi-endpoint bioassay system, we estimated the effects of chemicals on growth ability (IC50), viability (LD50), stress protein induction, prion mutation, mitochondrial mutation, and chromosomal mutation using the yeast Saccharomyces cerevisiae as a model organism. These endpoints showed characteristic values to 32 kinds of reference chemicals, and we classified the chemicals into seven groups according to the endpoints. We concluded that the yeast system has the potential to be a multi-endpoint bioassay relative to human health and the ecosystem in general.

L7 ANSWER 92 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 75
AN 2001:286410 BIOSIS Full-text
DN PREV200100286410
TI A new bioassay with the yeast Saccharomyces cerevisiae on aquatoxic pollution (Research Article).
Original Title: Ein neuer Biotest mit der Hefe Saccharomyces cerevisiae auf aquatische Toxizitaet.
AU Weber, Juergen; Plantikow, Anita; Kreutzmann, Jupp [Reprint author]
CS NORDUM Institut fuer Umwelt und Analytik GmbH and Co. KG, Am Weidenbruch 22, D-18196, Kessin/Rostock, Germany
info@nordum.de
SO Umweltwissenschaften und Schadstoff-Forschung, (2000) Vol. 12, No. 4, pp. 185-189. print.
ISSN: 0934-3504.
DT Article
LA German
ED Entered STN: 13 Jun 2001
Last Updated on STN: 19 Feb 2002
AB The fermentation activity of the yeast Saccharomyces cerevisiae is used as a bioindicator to detect aquatoxic effects. This is done by measuring the CO2 production of yeast cells after a multiplication period affected by toxic substances. The concentration of toxicants diminishing the fermentation by 20% is taken as a characteristic value (EG20). Organic compounds (unpolar and polar narcotics), inorganic salts (especially of heavy metals), surfactants and plant pesticides were tested. The results are compared so far as available with data of a ciliata assay using Tetrahymena pyriformis. A congruence of 90% and an equal sensibility of the assays resulted. The results of the yeast assay are therefore representative for ecotoxicological testing. The yeast assay is reproducible, easy to handle, and offers an alternative for sewage testing because sterile handling is not necessary.

L7 ANSWER 93 OF 184 MEDLINE ® on STN DUPLICATE 76
AN 2003041657 MEDLINE Full-text
DN PubMed ID: 12548888
TI Research of toxin and plasmids of Saccharomyces cerevisiae.
AU Qin Y; Gao D
CS Department of Microbiology, Shandong University, Jinan 250100.
SO Wei sheng wu xue bao = Acta microbiologica Sinica, (2000 Feb) Vol. 40, No. 1, pp. 105-7.
Journal code: 21610860R. ISSN: 0001-6209. L-ISSN: 0001-6209.
CY China
DT (ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Chinese
FS MEDLINE; Priority Journals
EM 200408
ED Entered STN: 29 Jan 2003
Last Updated on STN: 17 Dec 2003
Entered Medline: 10 Aug 2004
AB Killer toxin from Saccharomyces cerevisiae SK was isolated by ultrafiltration of culture supernatants and purified by poly(ethylene glycol). The toxin migrates as one single protein band on SDS-PAGE and its molecular weight is 15 kD. The SK toxin has the greatest lethal effect on the sensitive yeast strain in the lat-lag phase. Extraction and purification of killer heretity factor(dsRNA) from SK found that M-dsRNA plasmid and L-dsRNA plasmid have different molecular lengths being 1.7 kb and 4.0 kb.

L7 ANSWER 94 OF 184 MEDLINE ® on STN DUPLICATE 77
AN 2000156227 MEDLINE Full-text
DN PubMed ID: 10692229
TI Effect of stable integration of the Escherichia coli ada gene on the sensitivity of Saccharomyces cerevisiae to the toxic and mutagenic effects of alkylating agents.
AU Farkasova E; Chovanec M; Vlasakova D; Vickova V; Margison G P; Brozmanova J
CS Department of Molecular Genetics, Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovak Republic.

SO Environmental and molecular mutagenesis, (2000) Vol. 35, No. 1, pp. 66-9.
Journal code: 8800109. ISSN: 0893-6692. L-ISSN: 0893-6692.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200003
ED Entered STN: 7 Apr 2000
Last Updated on STN: 7 Apr 2000
Entered Medline: 24 Mar 2000

L7 ANSWER 95 OF 184 MEDLINE ® on STN DUPLICATE 78
AN 1999121035 MEDLINE Full-text
DN PubMed ID: 9922260
TI EmrE, a small Escherichia coli multidrug transporter, protects Saccharomyces cerevisiae from toxins by sequestration in the vacuole.
AU Yelin R; Rotem D; Schuldiner S
CS Alexander Silberman Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel.
NC NS16708 (United States NINDS NIH HHS)
SO Journal of bacteriology, (1999 Feb) Vol. 181, No. 3, pp. 949-56.
Journal code: 2985120R. ISSN: 0021-9193. L-ISSN: 0021-9193.
Report No.: NLM-PMC93463.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 199902
ED Entered STN: 11 Mar 1999
Last Updated on STN: 11 Mar 1999
Entered Medline: 23 Feb 1999
OSC.G 4 There are 4 MEDLINE records that cite this record
REM.CNT 44 There are 44 cited references available in MEDLINE for this document.
AB In this report we describe the functional expression of EmrE, a 110-amino-acid multidrug transporter from Escherichia coli, in the yeast Saccharomyces cerevisiae. To allow for phenotypic complementation, a mutant strain sensitive to a series of cationic lipophilic drugs was first identified. A hemagglutinin epitope-tagged version of EmrE (HA-EmrE) conferring resistance to a wide variety of drugs, including acriflavine, ethidium, methyl viologen, and the neurotoxin 1-methyl-4-phenylpyridinium (MPP+), was functionally expressed in this strain. HA-EmrE is expressed in yeast at relatively high levels (0.5 mg/liter), is soluble in a mixture of organic solvents, and can be functionally reconstituted in proteoliposomes. In bacterial cells, EmrE removes toxic compounds by active transport through the plasma membrane, lowering their cytosolic concentration. However, yeast cells expressing HA-EmrE take up 14C-methyl viologen as well as control cells do. Thus, we investigated the basis of the enhanced resistance to the above compounds. Using Cu2+ ions or methylamine, we could selectively permeabilize the plasma membrane or deplete the proton electrochemical gradients across the vacuolar membrane, respectively. Incubation of yeast cells with copper ions caused an increase in 14C-methyl viologen uptake. In contrast, treatment with methylamine markedly diminished the extent of uptake. Conversely, the effect of Cu2+ and methylamine on a plasma membrane uptake system, proline, was essentially the opposite: while inhibited by the addition of Cu2+, it remained unaffected when cells were treated with methylamine. To examine the intracellular distribution of HA-EmrE, a functional chimera between HA-EmrE and the green fluorescent protein (HA-EmrE-GFP) was prepared. The pattern of HA-EmrE-GFP fluorescence distribution was virtually identical to that of the vacuolar marker FM 4-64, indicating that the transporter is found mainly in this organelle. Therefore, HA-EmrE protects yeast cells by lowering the cytoplasmic concentrations through removal of the toxin to the vacuole. This novel way of detoxification has been previously suggested to function in organisms in which a large vacuolar compartment exists. This report represents the first molecular description of such a mechanism.

L7 ANSWER 96 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1999:338857 BIOSIS Full-text
DN PREV199900338857
TI Role of oxidative damage in heavy metal toxicity towards Saccharomyces cerevisiae.
AU Smith, S. L. [Reprint author]; Avery, S. V. [Reprint author]
CS Georgia State University, Atlanta, GA, USA
SO Abstracts of the General Meeting of the American Society for Microbiology, (1999) Vol. 99, pp. 386. print.
Meeting Info.: 99th General Meeting of the American Society for Microbiology. Chicago, Illinois, USA. May 30-June 3, 1999. American Society for Microbiology.
ISSN: 1060-2011.
DT Conference; (Meeting)
Conference; Abstract; (Meeting Abstract)
Conference; (Meeting Poster)
LA English
ED Entered STN: 24 Aug 1999
Last Updated on STN: 24 Aug 1999

L7 ANSWER 97 OF 184 MEDLINE ® on STN DUPLICATE 79
AN 2000276702 MEDLINE Full-text
DN PubMed ID: 10816727
TI Interaction of Saccharomyces cerevisiae with gold: toxicity and accumulation.
AU Karamushka V I; Gadd G M
CS Institute of Biocolloid Chemistry, National Academy of Sciences of the Ukraine, Kyiv, Ukraine.
SO Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine, (1999 Dec) Vol. 12, No. 4, pp. 289-94.
Journal code: 9208478. ISSN: 0966-0844. L-ISSN: 0966-0844.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English

FS MEDLINE; Priority Journals
EM 200006
ED Entered STN: 6 Jul 2000
Last Updated on STN: 6 Jul 2000
Entered Medline: 26 Jun 2000
OSC.G 1 There are 1 MEDLINE records that cite this record
AB This paper examines the effects of ionic gold on Saccharomyces cerevisiae, as determined by long-term (growth in gold-containing media) and short-term interactions (H+ efflux activity). An increasing gold concentration inhibited growth and at < 0.2 mM Au, growth was not observed. Transmission electron microscopy revealed no differences in ultrastructure but fine electron dense particles were observed in unstained preparations from gold-containing medium. After glucose addition (to 10 mM) to starved suspensions of S. cerevisiae, glucose-dependent reduction of external pH occurred as the cells extruded protons. In the presence of increasing gold concentrations, the lag time before proton extrusion did not change but the rate and duration decreased significantly with a marked influence on proton efflux rate being observed at < or = 10 microM. Extension of preincubation time of yeast cells in gold-containing medium resulted in a decreasing proton efflux rate and colloidal phase formation in the cell suspensions, the time between gold addition and the beginning of colloidal phase formation depending on the gold concentration used. Both Ca and Mg enhanced the inhibitory effect of gold on the yeast cells with Ca showing a stronger inhibitory effect than Mg.

L7 ANSWER 98 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 2000:367695 BIOSIS Full-text
DN PREV200000367695
TI Killer toxin of Saccharomyces cerevisiae Y500-4L active against Fleischmann and Itaiquara commercial brands of yeast.
AU Soares, Giselle A. M. [Reprint author]; Sato, Helia H.
CS Departamento de Ciencia de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas - UNICAMP, Campinas, SP, Brazil
SO Revista de Microbiologia, (Jul.-Set, 1999) Vol. 30, No. 3, pp. 253-257. print.
CODEN: RMBGBP. ISSN: 0001-3714.
DT Article
LA English
ED Entered STN: 23 Aug 2000
Last Updated on STN: 8 Jan 2002
AB The strain Saccharomyces cerevisiae Y500-4L, previously selected from the must of alcohol producing plants and showing high fermentative and killer capacities, was characterized according to the interactions between the yeasts and examined for curing and detection of dsRNA plasmids, which code for the killer character. The killer yeast S. cerevisiae Y500-4L showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against the standard killer yeasts K2 (S. diastaticus NCYC 713), K4 (Candida glabrata NCYC 388) and K11 (Torulopsis glabrata ATCC 15126). However S. cerevisiae Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K8 (Hansenula anomala NCYC 435), K9 (Hansenula mrakii NCYC 500), K10 (Kluyveromyces drosophilarum NCYC 575) and K11 (Torulopsis glabrata ATCC 15126). No M-dsRNA plasmid was detected in the S. cerevisiae Y500-4L strain and these results suggest that the genetic basis for toxin production is encoded by chromosomal DNA. The strain S. cerevisiae Y500-4L was more resistant to the loss of the phenotype killer with cycloheximide and incubation at elevated temperatures (40degreeC) than the standard killer yeast S. cerevisiae K1.

L7 ANSWER 99 OF 184 MEDLINE ® on STN DUPLICATE 80
AN 1999383903 MEDLINE Full-text
DN PubMed ID: 10452956
TI Expression of human inositol monophosphatase suppresses galactose toxicity in Saccharomyces cerevisiae: possible implications in galactosemia.
AU Mehta D V; Kabir A; Bhat P J
CS Laboratory of Molecular Genetics, Biotechnology Centre, Indian Institute of Technology, Powai, Mumbai 400 076, India.
SO Biochimica et biophysica acta, (1999 Aug 30) Vol. 1454, No. 3, pp. 217-26.
Journal code: 0217513. ISSN: 0006-3002. L-ISSN: 0006-3002.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199910
ED Entered STN: 14 Oct 1999
Last Updated on STN: 3 Mar 2000
Entered Medline: 1 Oct 1999
OSC.G 5 There are 5 MEDLINE records that cite this record
AB A suppressor of galactose toxicity in a gal7 yeast strain (lacking galactose 1-phosphate uridyl transferase) has been isolated from a HeLa cell cDNA library. Analysis of the plasmid clone indicated that the insert has an ORF identical to that of hIMPase (human myo-inositol monophosphatase). The ability of hIMPase to suppress galactose toxicity is sensitive to the presence of Li(+) in the medium. A gal7 yeast strain harboring a plasmid containing cloned hIMPase grows on galactose as a sole carbon source. hIMPase mediated galactose metabolism is dependent on the functionality of GAL1 as well as GAL10 encoded galactokinase and epimerase respectively. These results predicted that the UDP-glucose/galactose pyrophosphorylase mediated pathway may be responsible for the relief of galactose toxicity. Experiments conducted to test this prediction revealed that expression of UGP1 encoded UDP-glucose pyrophosphorylase can indeed overcome the relief of galactose toxicity. Moreover, expression of UGP1 allows a gal7 strain to grow on galactose as a sole carbon source. Unlike the hIMPase mediated relief of galactose toxicity, UGP1 mediated relief of galactose toxicity is lithium insensitive. Based on our results and on the basis of available information on galactose toxicity, we suggest an alternative explanation for the molecular mechanism of galactose toxicity.

L7 ANSWER 100 OF 184 MEDLINE ® on STN DUPLICATE 81
AN 2005124518 MEDLINE Full-text
DN PubMed ID: 9684308
TI Manganese toxicity towards Saccharomyces cerevisiae: dependence on intracellular and extracellular magnesium concentrations.
AU Blackwell K J; Tobin J M; Avery S V
CS School of Biological Sciences, Dublin City University, Dublin 9, Ireland.
SO Applied microbiology and biotechnology, (1998 Jun) Vol. 49, No. 6, pp. 751-7.
Journal code: 8406612. ISSN: 0175-7598. L-ISSN: 0175-7598.
CY Germany: Germany, Federal Republic of

DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 200503
ED Entered STN: 10 Mar 2005
Last Updated on STN: 31 Mar 2005
Entered Medline: 30 Mar 2005
OSC.G 2 There are 2 MEDLINE records that cite this record
AB Inhibition of the growth of Saccharomyces cerevisiae was evident at concentrations of 0.5 mM Mn2+ or higher, but a tolerance to lower Mn2+ concentrations was observed. The inhibitory effects of 2.0 mM Mn2+ were eliminated by supplementing the medium with excess Mg2+ (10 mM), whereas addition of excess Ca2+ and K+ had negligible effect on Mn2+ toxicity. Growth inhibition by Mn2+, in the absence of a Mg2+ supplement, was attributed to Mn2+ accumulation to toxic intracellular levels. Mn levels in S. cerevisiae grown in Mg(2+)-supplemented medium were severalfold lower than those of cells growing in unsupplemented medium. Mn2+ toxicity was also influenced by intracellular Mg, as Mn2+ toxicity was found to be more closely correlated with the cellular Mg:Mn ratio than with cellular Mn levels alone. Cells with low intracellular levels of Mg were more susceptible to Mn2+ toxicity than cells with high cellular Mg, even when sequestered Mn2+ levels were similar. A critical Mg:Mn ratio of 2.0 was identified below which Mn2+ toxicity became acute. The results demonstrate the importance of intracellular and extracellular competitive interactions in determining the toxicity of Mn2+.

L7 ANSWER 101 OF 184 MEDLINE ® on STN DUPLICATE 82
AN 1998244387 MEDLINE Full-text
DN PubMed ID: 9584988
TI Menadione toxicity in Saccharomyces cerevisiae cells: activation by conjugation with glutathione.
AU Zadzinski R; Fortuniak A; Bilinski T; Grey M; Bartosz G
CS Department of Molecular Biophysics, University of Lodz, Poland.
SO Biochemistry and molecular biology international, (1998 Apr) Vol. 44, No. 4, pp. 747-59.
Journal code: 9306673. ISSN: 1039-9712. L-ISSN: 1039-9712.
CY Australia
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199806
ED Entered STN: 25 Jun 1998
Last Updated on STN: 25 Jun 1998
Entered Medline: 12 Jun 1998
OSC.G 6 There are 6 MEDLINE records that cite this record
AB Menadione (2-methyl-1,4-naphthoquinone) has been used extensively as an oxidant stressor at the cellular level. However, the mechanism of cytotoxicity of this compound still remains controversial. This study deals with the role of intracellular glutathione in the resistance of the yeast Saccharomyces cerevisiae to menadione. Incubation with 0.5 mM menadione resulted in a decrease of total glutathione concentration in yeast cells, intracellular formation of menadione S-glutathione conjugate and export of the conjugate from cells. GSH-deficient mutants showed lower stimulation of superoxide and hydrogen peroxide production upon exposure to menadione and were more resistant to menadione than wild-type isogenic strains. These results indicate that in yeast cells the formation of S-glutathione conjugate is a major pathway of menadione metabolism and that this reaction leads to redox activation of menadione but permits its removal from the cells.

L7 ANSWER 102 OF 184 MEDLINE ® on STN DUPLICATE 83
AN 1998125670 MEDLINE Full-text
DN PubMed ID: 9464394
TI Intracellular signal triggered by cholera toxin in Saccharomyces boulardii and Saccharomyces cerevisiae.
AU Brandao R L; Castro I M; Bambirra E A; Amaral S C; Fietto L G; Tropia M J; Neves M J; Dos Santos R G; Gomes N C; Nicoli J R
CS Laboratorio de Fisiologia e Bioquimica de Microorganismos, Escola de Farmacia, Universidade Federal de Ouro Preto, Brazil.
SO Applied and environmental microbiology, (1998 Feb) Vol. 64, No. 2, pp. 564-8.
Journal code: 7605801. ISSN: 0099-2240. L-ISSN: 0099-2240.
Report No.: NLM-PMC106083.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199802
ED Entered STN: 6 Mar 1998
Last Updated on STN: 6 Mar 1998
Entered Medline: 26 Feb 1998
OSC.G 5 There are 5 MEDLINE records that cite this record
REM.CNT 17 There are 17 cited references available in MEDLINE for this document.
AB As is the case for Saccharomyces boulardii, Saccharomyces cerevisiae W303 protects Fisher rats against cholera toxin (CT). The addition of glucose or dinitrophenol to cells of S. boulardii grown on a nonfermentable carbon source activated trehalase in a manner similar to that observed for S.cerevisiae. The addition of CT to the same cells also resulted in trehalase activation. Experiments performed separately on the A and B subunits of CT showed that both are necessary for activation. Similarly, the addition of CT but not of its separate subunits led to a cyclic AMP (cAMP) signal in both S. boulardii and S. cerevisiae. These data suggest that trehalase stimulation by CT probably occurred through the cAMP-mediated protein phosphorylation cascade. The requirement of CT subunit B for both the cAMP signal and trehalase activation indicates the presence of a specific receptor on the yeasts able to bind to the toxin, a situation similar to that observed for mammalian cells. This hypothesis was reinforced by experiments with 125I-labeled CT showing specific binding of the toxin to yeast cells. The adhesion of CT to a receptor on the yeast surface through the B subunit and internalization of the A subunit (necessary for the cAMP signal and trehalase activation) could be one more mechanism explaining protection against the toxin observed for rats treated with yeasts.

L7 ANSWER 103 OF 184 MEDLINE ® on STN DUPLICATE 84
AN 1999059462 MEDLINE Full-text
DN PubMed ID: 9845329

TI C-terminal truncation of yeast SerRS is toxic for Saccharomyces cerevisiae due to altered mechanism of substrate recognition.
AU Lenhard B; Praetorius-Ibba M; Filipic S; Soll D; Weygand-Durasevic I
CS Department of Chemistry, Faculty of Science, University of Zagreb, Croatia.
SO FEBS letters, (1998 Nov 20) Vol. 439, No. 3, pp. 235-40.
Journal code: 0155157. ISSN: 0014-5793. L-ISSN: 0014-5793.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 199812
ED Entered STN: 15 Jan 1999
Last Updated on STN: 15 Jan 1999
Entered Medline: 21 Dec 1998
OSC.G 1 There are 1 MEDLINE records that cite this record
AB Like all other eukaryal cytosolic seryl-tRNA synthetase (SerRS) enzymes, Saccharomyces cerevisiae SerRS contains a C-terminal extension not found in the enzymes of eubacterial and archaeal origin. Overexpression of C-terminally truncated SerRS lacking the 20-amino acid appended domain (SerRSC20) is toxic to S. cerevisiae possibly because of altered substrate recognition. Compared to wild-type SerRS the truncated enzyme displays impaired tRNA-dependent serine recognition and is less stable. This suggests that the C-terminal peptide is important for the formation or maintenance of the enzyme structure optimal for substrate binding and catalysis.

L7 ANSWER 104 OF 184 MEDLINE ® on STN DUPLICATE 85
AN 1998057935 MEDLINE Full-text
DN PubMed ID: 9396148
TI Influence of altered plasma membrane fatty acid composition on cesium transport characteristics and toxicity in Saccharomyces cerevisiae.
AU Hoptroff M J; Thomas S; Avery S V
CS Department of Biology, Georgia State University, Atlanta 30303, USA.
SO Canadian journal of microbiology, (1997 Oct) Vol. 43, No. 10, pp. 954-62.
Journal code: 0372707. ISSN: 0008-4166. L-ISSN: 0008-4166.
CY Canada
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199801
ED Entered STN: 6 Feb 1998
Last Updated on STN: 6 Feb 1998
Entered Medline: 27 Jan 1998
OSC.G 1 There are 1 MEDLINE records that cite this record
AB The influence of altered plasma membrane fatty acid composition on cesium uptake and toxicity was investigated in Saccharomyces cerevisiae. Detailed kinetic studies revealed that both the Vmax and Km values for Cs+ transport increased (by approximately twofold in the latter case) when S. cerevisiae was grown in medium supplemented with the polyunsaturated fatty acid linoleate. In addition, Cs+ uptake by linoleate-enriched cells was considerably less sensitive to the competitive effects of other monovalent cations (K+, Rb+, and NH4+) than that by unsupplemented cells. Stimulation of Cs+ uptake in the presence of certain K+ and Rb+ concentrations was only evident in linoleate-enriched S. cerevisiae. At 100 mM CsCl, the initial rate of Cs+ uptake was greater in linoleate-supplemented cells than in unsupplemented cells and this was reflected in a more rapid displacement of cellular K+. However, little difference in net Cs+ accumulation between linoleate-supplemented and unsupplemented cells was evident during prolonged incubation in buffer or during growth. Thus, Cs+ toxicity was similar in linoleate-supplemented and unsupplemented cells. The results were consistent with the Cs+ (K+) transport mechanism adopting an altered conformational state in linoleate-enriched S. cerevisiae.

L7 ANSWER 105 OF 184 MEDLINE ® on STN DUPLICATE 86
AN 1997055696 MEDLINE Full-text
DN PubMed ID: 8899983
TI Copper toxicity towards Saccharomyces cerevisiae: dependence on plasma membrane fatty acid composition.
AU Avery S V; Howlett N G; Radice S
CS School of Biological and Molecular Sciences, Oxford Brookes University, Headington, United Kingdom.
SO Applied and environmental microbiology, (1996 Nov) Vol. 62, No. 11, pp. 3960-6.
Journal code: 7605801. ISSN: 0099-2240. L-ISSN: 0099-2240.
Report No.: NLM-PMC168214.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199701
ED Entered STN: 28 Jan 1997
Last Updated on STN: 6 Feb 1998
Entered Medline: 8 Jan 1997
OSC.G 14 There are 14 MEDLINE records that cite this record
REM.CNT 23 There are 23 cited references available in MEDLINE for this document.
AB One major mechanism of copper toxicity towards microorganisms is disruption of plasma membrane integrity. In this study, the influence of plasma membrane fatty acid composition on the susceptibility of Saccharomyces cerevisiae to Cu2+ toxicity was investigated. Microbial fatty acid composition is highly variable, depending on both intrinsic and environmental factors. Manipulation was achieved in this study by growth in fatty acid-supplemented medium. Whereas cells grown under standard conditions contained only saturated and monounsaturated fatty acids, considerable incorporation of the diunsaturated fatty acid linoleate (18:2) (to more than 65% of the total fatty acids) was observed in both whole-cell homogenates and plasma membrane-enriched fractions from cells grown in linoleate-supplemented medium. Linoleate enrichment had no discernible effect on the growth of S. cerevisiae. However, linoleate-enriched cells were markedly more susceptible to copper-induced plasma membrane permeabilization. Thus, after addition of Cu(NO3)2, rates of cellular K+ release (loss of membrane integrity) were at least twofold higher from linoleate-supplemented cells than from unsupplemented cells; this difference increased with reductions in the Cu2+ concentration supplied. Levels of cellular Cu accumulation were also higher in linoleate-supplemented cells. These results were

correlated with a very marked dependence of whole-cell Cu2+ toxicity on cellular fatty acid unsaturation. For example, within 10 min of exposure to 5 microM Cu2+, only 3% of linoleate-enriched cells remained viable (capable of colony formation). In contrast, 100% viability was maintained in cells previously grown in the absence of a fatty acid supplement. Cells displaying intermediate levels of linoleate incorporation showed intermediate Cu2+ sensitivity, while cells enriched with the triunsaturated fatty acid linolenate (18:3) were most sensitive to Cu2+. These results demonstrate for the first time that changes in cellular and plasma membrane fatty acid compositions can dramatically alter microbial sensitivity to copper.

L7 ANSWER 106 OF 184 MEDLINE ® on STN DUPLICATE 87
AN 1997071674 MEDLINE Full-text
DN PubMed ID: 8914520
TI Gene isolation in Arabidopsis thaliana by conditional overexpression of cDNAs toxic to Saccharomyces cerevisiae: identification of a novel early response zinc-finger gene.
AU Martinez-Garcia M; Garciduenas-Pina C; Guzman P
CS Departamento de Ingenieria Genetica de Plantas, Centro de Investigacion y de Estudios Avanzados del IPN, Irapuato, GTO., Mexico.
SO Molecular & general genetics : MGG, (1996 Oct 16) Vol. 252, No. 5, pp. 587-96.
Journal code: 0125036. ISSN: 0026-8925. L-ISSN: 0026-8925.
CY GERMANY: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals; Space Life Sciences
OS GENBANK-L76926
EM 199612
ED Entered STN: 28 Jan 1997
Last Updated on STN: 6 Feb 1998
Entered Medline: 16 Dec 1996
OSC.G 6 There are 6 MEDLINE records that cite this record
AB In an effort to identify novel regulatory plant genes, conditional overexpression of toxic Arabidopsis thaliana gene products in Saccharomyces cerevisiae was evaluated as a genetic selection scheme. The screening method was tested on a fraction of a cDNA expression library and led to the identification of two Arabidopsis cDNA clones that were toxic to yeast; one corresponded to histone H1 and the other to a previously unidentified gene. This new gene, named ATL2, combines a RING-like zinc-binding motif and a putative signal anchor sequence for membrane insertion in the same molecule. Furthermore, inspection of the 3' untranslated region reveals two types of sequences which appear to be key determinants in rapid transcript decay. Indeed, rapid and transient accumulation of transcript occurs in the presence of a protein synthesis inhibitor and of the growth regulator auxin. These features provide evidence that ATL2 is an early-response gene. Thus, ATL2 represents one of the first early-response plant genes to be described which possesses a distinct regulatory domain; the fact that ATL2 mRNA is induced by auxin suggests that it might have a role during the response of plants to this growth regulator.

L7 ANSWER 107 OF 184 MEDLINE ® on STN DUPLICATE 88
AN 1997093966 MEDLINE Full-text
DN PubMed ID: 8939429
TI Highly conserved toxicity of Saccharomyces cerevisiae Rap1p.
AU Chambers A
CS Department of Genetics, University of Nottingham, Queen's Medical Centre, UK. Alistair.Chambers@nottingham.ac.uk
SO Molecular microbiology, (1996 Nov) Vol. 22, No. 3, pp. 449-58.
Journal code: 8712028. ISSN: 0950-382X. L-ISSN: 0950-382X.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199703
ED Entered STN: 21 Mar 1997
Last Updated on STN: 21 Mar 1997
Entered Medline: 13 Mar 1997
OSC.G 1 There are 1 MEDLINE records that cite this record
AB Budding yeast (Saccharomyces cerevisiae) Rap1p has been expressed in fission yeast (Schizosaccharomyces pombe) under the control of the regulatable fructose bisphosphatase (fbp) promoter. When the fbp promoter was derepressed, cells containing the complete RAP1 gene failed to show any significant growth, suggesting that Rap1p is toxic. A derivative of Rap1p that has a temperature-sensitive mutation in the DNA-binding domain was not toxic in cells grown at 37 degrees C, a temperature at which DNA binding by rap1p(ts) is severely inhibited. Removal of a short region downstream of the DNA-binding domain, including a region previously shown to be essential for Rap1p toxicity in budding yeast, also abolished the toxic effect. The toxic effect of Rap1p has therefore been conserved between two distantly related yeasts. In budding yeast, overexpression of Rap1p also caused changes to the lengths of the telomeric repeats. No effects on telomeres were detected in fission yeast.

L7 ANSWER 108 OF 184 MEDLINE ® on STN DUPLICATE 89
AN 1996240633 MEDLINE Full-text
DN PubMed ID: 8659103
TI Proteolytically active 2A proteinase of human rhinovirus 2 is toxic for Saccharomyces cerevisiae but does not cleave the homologues of eIF-4 gamma in vivo or in vitro.
AU Klump H; Auer H; Liebig H D; Kuechler E; Skern T
CS Department of Biochemistry, Medical Faculty, University of Vienna, Austria.
SO Virology, (1996 Jun 1) Vol. 220, No. 1, pp. 109-18.
Journal code: 0110674. ISSN: 0042-6822. L-ISSN: 0042-6822.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199608
ED Entered STN: 8 Aug 1996
Last Updated on STN: 3 Mar 2000
Entered Medline: 1 Aug 1996

OSC.G 6 There are 6 MEDLINE records that cite this record
AB During the replication of rhino- and enteroviruses, the translation initiation factor eIF-4 gamma is specifically cleaved by the virally encoded 2 A proteinase. This cleavage has been proposed to lead to the inability of the host cell to translate its own capped mRNA and to stimulate internal initiation of protein synthesis from the viral mRNA. However, a direct causal relationship between these effects and 2A proteinase-mediated cleavage of eIF-4 gamma has remained difficult to prove, mainly because of the toxicity of the 2A proteinase in mammalian expression systems. As an alternative approach, we placed the cDNA sequences for the human rhinovirus 2 2A proteinase and two mutants defective in proteolytic activity under the control of an inducible yeast Gal1-10 promoter and stably integrated them into the yeast genome. Induction of the wildtype enzyme led to changes in cellular morphology, an inhibition of cell division activity, and finally to cell death. As the yeast homologues of mammalian eIF-4 gamma, p150 and p130, were shown to be refractory to cleavage by human rhinovirus 2A proteinase both in vivo and in vitro and the rate of protein synthesis was unaffected, the toxicity of the 2A proteinase toward budding yeast must be due to its interaction with at least one other cellular protein essential for viability.

L7 ANSWER 109 OF 184 MEDLINE ® on STN DUPLICATE 90
AN 1997246267 MEDLINE Full-text
DN PubMed ID: 9090828
TI The target of sodium toxicity in Saccharomyces cerevisiae.
AU Almagro A; Gomez M J; Ramos J
CS Departamento de Microbiologia, Escuela Tecnica Superior de Ingenieros Agronomos y Montes, Cordoba, Espana.
SO Folia microbiologica, (1996) Vol. 41, No. 1, pp. 82-3.
Journal code: 0376757. ISSN: 0015-5632. L-ISSN: 0015-5632.
CY Czech Republic
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199705
ED Entered STN: 23 May 1997
Last Updated on STN: 31 Oct 2002
Entered Medline: 14 May 1997

L7 ANSWER 110 OF 184 MEDLINE ® on STN DUPLICATE 91
AN 1996258431 MEDLINE Full-text
DN PubMed ID: 8777437
TI Cell wall synthesis specific cytocidal effect of Hansenula mrakii toxin-1 on Saccharomyces cerevisiae.
AU Takasuka T; Komiyama T; Furuichi Y; Watanabe T
CS Department of Molecular Genetics, Nippon Roche Research Center, Kamakura, Japan.
SO Cellular & molecular biology research, (1995) Vol. 41, No. 6, pp. 575-81.
Journal code: 9316986. ISSN: 0968-8773. L-ISSN: 0968-8773.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199609
ED Entered STN: 24 Sep 1996
Last Updated on STN: 24 Sep 1996
Entered Medline: 13 Sep 1996
OSC.G 5 There are 5 MEDLINE records that cite this record
AB HM-1 toxin produced by Hansenula mrakii kills sensitive Saccharomyces cerevisiae. We found that the budding cells and the cells that responded to mating factor were sensitive to HM-1 toxin. These findings indicate that the target sites of HM-1 toxin are developing buds and conjugating tubes. The in vitro activity of beta-1,3-glucan synthase solubilized and partially purified from S. cerevisiae membranes was inhibited by HM-1 toxin at a concentration (around 50 nM, 0.5 micrograms/ml) that coincided well with its minimum inhibitory concentration for the growth of yeast cells. These data indicate that the HM-1 toxin perturbs the synthesis of yeast cell walls by inhibiting the glucan synthesis occurring at a budding site or a conjugating tube, which results in cell lysis.

L7 ANSWER 111 OF 184 MEDLINE ® on STN DUPLICATE 92
AN 1995122177 MEDLINE Full-text
DN PubMed ID: 7822013
TI Pathogenicity of Saccharomyces cerevisiae in complement factor five-deficient mice.
AU Byron J K; Clemons K V; McCusker J H; Davis R W; Stevens D A
CS Department of Biology, Stanford University, California.
NC AI 27076 (United States NIAID NIH HHS)
SO Infection and immunity, (1995 Feb) Vol. 63, No. 2, pp. 478-85.
Journal code: 0246127. ISSN: 0019-9567. L-ISSN: 0019-9567.
Report No.: NLM-PMC173020.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 199502
ED Entered STN: 23 Feb 1995
Last Updated on STN: 23 Feb 1995
Entered Medline: 16 Feb 1995
OSC.G 14 There are 14 MEDLINE records that cite this record
REM.CNT 27 There are 27 cited references available in MEDLINE for this document.
AB We have previously determined the relative virulence of isolates of Saccharomyces cerevisiae on the basis of differences in proliferation and resistance to clearance in CD-1 mice. These infections were not fatal. To further characterize S. cerevisiae pathogenesis, we studied a virulent clinical isolate, YJM128, and an avirulent nonclinical isolate, Y55, in C5-deficient mice. DBA/2N mice were infected intravenously with YJM128 or Y55, and temporal burdens of yeast cells in various organs were determined. After infection with 10(7) CFU, Y55 increased by 13-fold and YJM128 increased by 20-fold in the brain from day 0 to 3. In addition, YJM128 increased by 4-fold in the kidneys, whereas Y55 decreased by 16-fold. Both isolates declined in number in other organs. In all studies, 90% of mice infected with 10(7) CFU of YJM128 died between days 2 and 7, whereas no mice infected with equivalent numbers of Y55 died. No mice

died after infection with 10(6) CFU of Y55 or YJM128. The importance of C5 was confirmed by studies using B10.D2/oSnJ (C5-) mice and their congenic C5+ counterparts. Again, the C5- mice were most susceptible to infection with S. cerevisiae, with 63% infected with YJM128 dying by day 7; no C5+ mice died. No Y55-infected mice died, and mean burdens in the brain at day 14 were sevenfold lower in C5+ mice than in C5- mice. Seven of 10 other S. cerevisiae isolates were also more virulent in DBA/2N than CD-1 mice, causing > or = 40% mortality. These data indicate that C5 is a critical factor in host resistance against S. cerevisiae infections and further confirm the pathogenic potential of some isolates of S. cerevisiae.

L7 ANSWER 112 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 93
AN 1996:59391 BIOSIS Full-text
DN PREV199698631526
TI Production of HM-1 killer toxin in Saccharomyces cerevisiae transformed with the PDR4 gene and delta-sequence-mediated multi-integration system.
AU Kimura, Tetsuya [Reprint author]; Kitamoto, Noriyuki; Iimura, Yuzuru; Kito, Yukio
CS Sch. Bioresources, Mie Univ., Kamihama-cho, Tsu 514, Japan
SO Journal of Fermentation and Bioengineering, (1995) Vol. 80, No. 5, pp. 423-428.
CODEN: JFBIEX. ISSN: 0922-338X.
DT Article
LA English
ED Entered STN: 9 Feb 1996
Last Updated on STN: 9 Feb 1996
AB The gene for the HM-1 killer toxin of Williopsis mrakii IFO 0895, which has a broad killer spectrum and is stable over wide pH and temperature ranges, was introduced into a killer-resistant mutant of Saccharomyces cerevisiae using a 2 mu-m plasmid or delta-sequence-mediated multi-integration system under the control of the ADH1 promoter together with the PDR4 gene as a dominant selective marker. The killer activity of the transformants harboring the killer gene on a YEp plasmid was mitotically unstable and the plasmid copy number was reduced on a nonselective medium. In contrast, some transformants obtained using the delta-integration system showed strong killer activity which was mitotically stable even on a non-selective medium. Integration of multiple copies of the killer gene was confirmed by genomic Southern blot analysis. In a mixed culture experiment of the transformant using delta-mediated multi-integration which produces HM-1 and a HM-1-sensitive strain, Pichia anomala IFO 0569, the transformant prevented the growth of the latter.

L7 ANSWER 113 OF 184 MEDLINE ® on STN DUPLICATE 94
AN 1994245322 MEDLINE Full-text
DN PubMed ID: 8188341
TI Intracellular expression of toxic shock syndrome toxin 1 in Saccharomyces cerevisiae.
AU Deresiewicz R L; Flaxenburg J A; Chan M; Finberg R W; Kasper D L
CS Channing Laboratory, Boston, MA 02115.
SO Infection and immunity, (1994 Jun) Vol. 62, No. 6, pp. 2202-7.
Journal code: 0246127. ISSN: 0019-9567. L-ISSN: 0019-9567.
Report No.: NLM-PMC186498.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199406
ED Entered STN: 29 Jun 1994
Last Updated on STN: 29 Jun 1994
Entered Medline: 23 Jun 1994
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 19 There are 19 cited references available in MEDLINE for this document.
AB In order to search for an occult cytotoxic enzymatic activity of the toxic shock syndrome toxin 1 (TSST-1), we placed the gene encoding TSST-1 (tstH) under the control of an inducible promoter in the eukaryotic yeast Saccharomyces cerevisiae. Under similar circumstances, the known bacterial enzymatic cytotoxins Shiga-like toxin and diphtheria toxin are both highly lethal to the yeast host. Although full-length stable TSST-1 was demonstrated within the yeast cells and although it retained mitogenicity for human T cells, it had no apparent effect on the yeast cells' growth kinetics or on their gross morphology. Retrieval and sequencing of the toxin gene revealed the wild-type sequence throughout, thus demonstrating that the apparent lack of toxicity for the yeast cells was not due to a serendipitous attenuating mutation within the coding region of the toxin gene. Similar results obtained after a second transformation of the same strain and after transformation of an unrelated strain demonstrate that neither chance permissive host mutation nor intrinsic host resistance was likely to have obscured an existing cytotoxic property of TSST-1. We conclude that TSST-1 probably does not possess a discrete enzymatic property cytotoxic for eukaryotic cells.

L7 ANSWER 114 OF 184 MEDLINE ® on STN DUPLICATE 95
AN 1994283891 MEDLINE Full-text
DN PubMed ID: 8013903
TI Genetic characterization of pathogenic Saccharomyces cerevisiae isolates.
AU McCusker J H; Clemons K V; Stevens D A; Davis R W
CS Department of Biochemistry, Stanford University School of Medicine, California 94305.
NC AI 27076 (United States NIAID NIH HHS)
SO Genetics, (1994 Apr) Vol. 136, No. 4, pp. 1261-9.
Journal code: 0374636. ISSN: 0016-6731. L-ISSN: 0016-6731.
Report No.: NLM-PMC1205906.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 199407
ED Entered STN: 10 Aug 1994
Last Updated on STN: 10 Aug 1994
Entered Medline: 25 Jul 1994

OSC.G 40 There are 40 MEDLINE records that cite this record
REM.CNT 16 There are 16 cited references available in MEDLINE for this document.

AB Saccharomyces cerevisiae isolates from human patients have been genetically analyzed. Some of the characteristics of these isolates are very different from laboratory and industrial strains of S. cerevisiae and, for this reason, stringent genetic tests have been used to confirm their identity as S. cerevisiae. Most of these clinical isolates are able to grow at 42 degrees, a temperature that completely inhibits the growth of most other S. cerevisiae strains. This property can be considered a virulence trait and may help explain the presence of these isolates in human hosts. The ability to grow at 42 degrees is shown to be polygenic with primarily additive effects between loci. S. cerevisiae will be a useful model for the evolution and genetic analysis of fungal virulence and the study of polygenic traits.

L7 ANSWER 115 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 96
AN 1994:554226 BIOSIS Full-text
DN PREV199598013774
TI Overexpressed RAD4 Protein Required for Excision Repair of Saccharomyces cerevisiae is Toxic to the Host Escherichia coli.
AU Kim, J. B.; Jeon, S. H.; Choi, I. S.; Park, S. D. [Reprint author]
CS Dep. Mol. Biol., Seoul Natl. Univ., Seoul 151-742, South Korea
SO In Vitro Toxicology, (1994) Vol. 7, No. 3, pp. 269-275.
CODEN: IVTOE4. ISSN: 0888-319X.
DT Article
LA English
ED Entered STN: 22 Dec 1994
Last Updated on STN: 22 Dec 1994

AB The RAD4 gene of Saccharomyces cerevisiae, which is absolutely required for nucleotide excision repair, was overexpressed in E. coli BL21/DE3 cells. The overproduced Rad4 protein appeared to be toxic to the host Escherichia coli. Two-dimensional gel electrophoresis showed that a number of other protein spots dramatically disappeared when the Rad4 protein was overexpressed. The growth kinetics of E. coli containing Rad4 protein showed a remarkable delay in growth rate. To determine which part of RAD4 gene contributes to the toxicity in E. coli, various fusion plasmids containing a partial sequence of RAD4 and lac'Z genes were constructed. After induction with mitomycin C, the transformants containing the C-terminus of RAD4 gene fused with lac'Z gene were transcribed normally, but little or no fusion protein was produced in these cells. These results suggest that the C-terminal domain of Rad4 protein may be important for both toxic effect in E. coli and for its role in DNA repair in S. cerevisiae.

L7 ANSWER 116 OF 184 MEDLINE ® on STN DUPLICATE 97
AN 1993216084 MEDLINE Full-text
DN PubMed ID: 8462845
TI A mutational analysis of killer toxin resistance in Saccharomyces cerevisiae identifies new genes involved in cell wall (1-->6)-beta-glucan synthesis.
AU Brown J L; Kossaczka Z; Jiang B; Bussey H
CS Biology Department, McGill University, Montreal, Quebec, Canada.
SO Genetics, (1993 Apr) Vol. 133, No. 4, pp. 837-49.
Journal code: 0374636. ISSN: 0016-6731. L-ISSN: 0016-6731.
Report No.: NLM-PMC1205404.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
OS GENBANK-L07812; GENBANK-L07813; GENBANK-L07814; GENBANK-L07815; GENBANK-L07816; GENBANK-L07817; GENBANK-L07818; GENBANK-L07819; GENBANK-L07820; GENBANK-L10667
EM 199305
ED Entered STN: 21 May 1993
Last Updated on STN: 7 Feb 2003
Entered Medline: 6 May 1993
OSC.G 28 There are 28 MEDLINE records that cite this record
REM.CNT 27 There are 27 cited references available in MEDLINE for this document.

AB Recessive mutations leading to killer resistance identify the KRE9, KRE10 and KRE11 genes. Mutations in both the KRE9 and KRE11 genes lead to reduced levels of (1-->6)-beta-glucan in the yeast cell wall. The KRE11 gene encodes a putative 63-kD cytoplasmic protein, and disruption of the KRE11 locus leads to a 50% reduced level of cell wall (1-->6)-glucan. Structural analysis of the (1-->6)-beta-glucan remaining in a kre11 mutant indicates a polymer smaller in size than wild type, but containing a similar proportion of (1-->6)- and (1-->3)-linkages. Genetic interactions among cells harboring mutations at the KRE11, KRE6 and KRE1 loci indicate lethality of kre11 kre6 double mutants and that kre11 is epistatic to kre1, with both gene products required to produce the mature glucan polymer at wild-type levels. Analysis of these KRE genes should extend knowledge of the beta-glucan biosynthetic pathway, and of cell wall synthesis in yeast.

L7 ANSWER 117 OF 184 MEDLINE ® on STN DUPLICATE 98
AN 1993230441 MEDLINE Full-text
DN PubMed ID: 8472187
TI IgE-binding components of baker's yeast (Saccharomyces cerevisiae) recognized by immunoblotting analysis. Simultaneous IgE binding to mannan and 46-48 kD allergens of Saccharomyces cerevisiae and Candida albicans.
AU Kortekangas-Savolainen O; Kalimo K; Lammintausta K; Savolainen J
CS Department of Dermatology, University of Turku, Finland.
SO Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, (1993 Mar) Vol. 23, No. 3, pp. 179-84.
Journal code: 8906443. ISSN: 0954-7894. L-ISSN: 0954-7894.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 199305
ED Entered STN: 4 Jun 1993
Last Updated on STN: 4 Jun 1993
Entered Medline: 20 May 1993

OSC.G 6 There are 6 MEDLINE records that cite this record
AB The Saccharomyces cerevisiae allergens were characterized by IgE-immunoblotting with serum samples of 83 patients; 63 represented patients with atopic dermatitis with previous positive skin prick test or RAST for S. cerevisiae, seven patients with AD but negative test results and 13 were non-atopic controls. Disrupted whole body extract of S. cerevisiae was used in the assays. From the patients tested 41 patients with atopic dermatitis appeared positive in IgE immunoblotting revealing 22 IgE stained bands. From these bands 10 represented intermediate allergens, and 12 minor allergens. The most frequent staining was obtained with the 48 kD band (39%). When the staining pattern of 45 kD and 48 kD bands and mannan was compared with Candida albicans allergens or purified baker's yeast enolase a simultaneous binding was seen with the 48 kD band of S. cerevisiae and the 46 kD band of C. albicans and enolase whereas the 45 kD band was neither associated with the 46 kD band of C. albicans nor purified enolase. High molecular weight staining was found in five samples. The staining pattern was associated with the mannose containing structures in parallel with C. albicans.

L7 ANSWER 118 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1993:251919 BIOSIS Full-text
DN PREV199395131094
TI A mutational analysis of killer toxin resistance in Saccharomyces cerevisiae identifies new genes involved in cell wall (1 fwdarw 6)-beta-glucan synthesis.
AU Brown, Jeffrey L. [Reprint author]; Kossaczka, Zuzana; Jiang, Bo [Reprint author]; Bussey, Howard [Reprint author]
CS Biol. Dep., McGill Univ., Montreal, Quebec, Canada H3A 1B1, canada
SO Genetics, (1993) Vol. 133, No. 4, pp. 837849.
CODEN: GENTAE. ISSN: 0016-6731.
DT Article
LA English
ED Entered STN: 21 May 1993
Last Updated on STN: 13 Jul 1993
AB Recessive mutations leading to killer resistance identify the KRE9, KRE10 and KRE11 genes. Mutations in both the KRE9 and KRE11 genes lead to reduced levels of (1 fwdarw 6)-beta-glucan in the yeast cell wall. The KRE11 gene encodes a putative 63-kD cytoplasmic protein, and disruption of the KRE11 locus leads to a 50% reduced level of cell wall (1 fwdarw 6)-glucan. Structural analysis of the (1 fwdarw 6)-beta-glucan remaining in a kre11 mutant indicates a polymer smaller in size than wild type, but containing a similar mutations at the KRE11, KRE6 and KRE1 loci indicate lethality of kre11 kre6 double mutants and that kre11 is epistatic to kre1, with both gene products required to produce the mature glucan polymer at wild-type levels. Analysis of these KRE genes should extend knowledge of the beta-glucan biosynthetic pathway, and of cell wall synthesis in yeast.

L7 ANSWER 119 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1993:154885 BIOSIS Full-text
DN PREV199344073685
TI Using a Saccharomyces cerevisiae for toxicity assessment including interacting effects and DNA damage.
AU Kungolos, A.; Aoyama, I.
CS Lab. Ecol. Chem., Res. Inst. Bioresources, Okayama Univ., 2-20-1 Chuo-o, Kurashiki 710, Japan
SO Water Science and Technology, (1992) Vol. 25, No. 11, pp. 309-316.
Meeting Info.: Meeting on Hazard Assessment and Control of Environmental Contaminants in Water held at the 1st IAWPRC (International Association on Water Pollution Research and Control). Otsu City, Shiga, Japan. November 25-28, 1991.
CODEN: WSTED4. ISSN: 0273-1223.
DT Article
Conference; (Meeting)
LA English
ED Entered STN: 19 Mar 1993
Last Updated on STN: 20 Mar 1993

L7 ANSWER 120 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 1993043535 EMBASE Full-text
TI Using Saccharomyces cerevisiae for toxicity assessment including interacting effects and DNA damage.
AU Kungolos, A. (correspondence); Aoyama, I.
CS Laboratory of Ecological Chemistry, Research Institute for Bioresources, Okayama University, 2-20-1 Chu-o, Kurashiki 710, Japan.
SO Water Science and Technology, (1992) Vol. 26, No. 12, pp. 309-316.
ISSN: 0273-1223 CODEN: WSTED4
CY United Kingdom
DT Journal; Conference Article; (Conference paper)
FS 004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
046 Environmental Health and Pollution Control
052 Toxicology
LA English
SL English
ED Entered STN: 7 Mar 1993
Last Updated on STN: 7 Mar 1993
AB Saccharomyces cerevisiae is used in this study not only as a test organism for routine quick toxicity screening but also as a potential indicator of DNA damage by chemicals. Several indices were investigated for evaluating the inhibition rates caused by some heavy metals. Specific growth rate proved to be the most suitable index. Of the chemicals used in this study CH3HgCl was the most toxic with an EC50 of 3.5 x 10-4 mmol/l. The EC50s for HgCl2. CdCl2 and NiCl2 were 0.192, 0.0345 and 2.30 mmol/l respectively, using specific growth rate as an index. The interacting effect between Cd and Ni was generally additive. In order to detect DNA damage the growth of wild type strains was compared with the growth of strains deficient in one or all the DNA repairing systems. A known mutagen, 4-nitroquinoneline 1-oxide, and a non-mutagen, kanamycin, were used. The experimental results confirmed that 4-nitroquinoneline 1-oxide damaged the DNA of Saccharomyces cerevisiae, while kanamycin did not damage it. The importance of this confirmation lies in the fact that this test uses Saccharomyces cerevisiae, which is a eucaryotic organism and closer to human being than the procaryotic organisms that are usually used in other mutagenicity tests such as Ames test and Rec-assay.

L7 ANSWER 121 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on

STN
AN 1992:381720 BIOSIS Full-text
DN PREV199243048670; BR43:48670
TI SULFITE TOXICITY IN THE YEAST SACCHAROMYCES-CEREVISIAE.
AU WIGHTMAN J D [Reprint author]; XU X; GELLER B L; BAKALINSKY A T
CS OREGON STATE UNIV, CORVALLIS, OREGON, USA
SO Abstracts of the General Meeting of the American Society for Microbiology, (1992) Vol. 92, pp. 259.
Meeting Info.: 92ND GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, NEW ORLEANS, LOUISIANA, USA, MAY 26-30, 1992. ABSTR GEN MEET AM SOC MICROBIOL.
ISSN: 1060-2011.
DT Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 17 Aug 1992
Last Updated on STN: 17 Aug 1992

L7 ANSWER 122 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1992:380357 BIOSIS Full-text
DN PREV199243047307; BR43:47307
TI GENETIC SELECTION OF ACTIVE-SITE MUTANTS OF DIPHTHERIA TOXIN FRAGMENT A IN SACCHAROMYCES-CEREVISIAE.
AU FU H [Reprint author]; MATTHEAKIS L C; COLLIER R J
CS HARVARD MEDICAL SCHOOL, BOSTON, MASS, USA
SO Abstracts of the General Meeting of the American Society for Microbiology, (1992) Vol. 92, pp. 30.
Meeting Info.: 92ND GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, NEW ORLEANS, LOUISIANA, USA, MAY 26-30, 1992. ABSTR GEN MEET AM SOC MICROBIOL.
ISSN: 1060-2011.
DT Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 17 Aug 1992
Last Updated on STN: 17 Aug 1992

L7 ANSWER 123 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1992:247651 BIOSIS Full-text
DN PREV199242117951; BR42:117951
TI ISOLATION AND CHARACTERIZATION OF DIPHTHERIA TOXIN-RESISTANT MUTANTS FROM SACCHAROMYCES-CEREVISIAE.
AU ISHIURA M [Reprint author]; IIDA H; OKADA Y
CS NATIONAL INSTITUTE BASIC BIOLOGY, OKAZAKI, AICHI 444
SO Cell Structure and Function, (1991) Vol. 16, No. 6, pp. 566.
Meeting Info.: FORTY-FOURTH ANNUAL MEETING OF THE JAPAN SOCIETY FOR CELL BIOLOGY, FUKUOKA, JAPAN, NOVEMBER 21-23, 1991. CELL STRUCT FUNCT.
CODEN: CSFUDY. ISSN: 0386-7196.
DT Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 14 May 1992
Last Updated on STN: 15 May 1992

L7 ANSWER 124 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1991:517626 BIOSIS Full-text
DN PREV199141118341; BR41:118341
TI EFFECT OF SACCHAROMYCES-BOULARDII ON TOXINS PRODUCED BY CLOSTRIDIUM-DIFFICILE.
AU LUCAS F [Reprint author]; CORTHIER G
CS LABORATOIRE D'ECOLOGIE PHYSIOLOGIE DU SYSTEME DIGESTIF, 78352 JOUY-EN-JOSAS, FRANCE
SO Microbial Ecology in Health and Disease, (1991) Vol. 4, No. 4, pp. 238.
Meeting Info.: XV INTERNATIONAL CONGRESS ON MICROBIAL ECOLOGY AND DISEASE.
MICROB ECOL HEALTH DIS.
ISSN: 0891-060X.
DT Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 14 Nov 1991
Last Updated on STN: 14 Nov 1991

L7 ANSWER 125 OF 184 MEDLINE ® on STN DUPLICATE 99
AN 1990368663 MEDLINE Full-text
DN PubMed ID: 2144280
TI Plasma membrane-stimulated vanadate-dependent NADH oxidation is not the primary mediator of vanadate toxicity in Saccharomyces cerevisiae.
AU Minasi L A; Chang A; Willsky G R
CS Department of Biochemistry, State University of New York School of Medicine and Biochemical Sciences, Buffalo 14214.
SO The Journal of biological chemistry, (1990 Sep 5) Vol. 265, No. 25, pp. 14907-10.
Journal code: 2985121R. ISSN: 0021-9258. L-ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 199010
ED Entered STN: 9 Nov 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 9 Oct 1990
OSC.G 2 There are 2 MEDLINE records that cite this record

AB Interactions of oxyvanadium compounds with cellular metabolism have recently been demonstrated. Membrane-stimulated vanadate-dependent NADH oxidation has been hypothesized to involve the cellular accumulation of H2O2, which may cause the vanadate sensitivity of animals and microbes. This report shows that the vanadate-dependent NADH oxidation activity of the yeast plasma membrane requires oxygen and is present in vanadate-resistant mutants of Saccharomyces cerevisiae. In addition, the vanadate sensitivity of growth in S. cerevisiae is the same during aerobic and anaerobic growth. These results imply that neither plasma membrane-mediated vanadate-stimulated NADH oxidation, nor any other oxidative process, is the primary cause of vanadate sensitivity in yeast cells.

L7 ANSWER 126 OF 184 MEDLINE ® on STN DUPLICATE 100
AN 1990205813 MEDLINE Full-text
DN PubMed ID: 2181272
TI Ustilago maydis KP6 killer toxin: structure, expression in Saccharomyces cerevisiae, and relationship to other cellular toxins.
AU Tao J; Ginsberg T; Banerjee N; Held W; Koltin Y; Bruenn J A
CS Department of Biological Sciences, State University of New York, Buffalo 14260.
NC GM22200 (United States NIGMS NIH HHS)
SO Molecular and cellular biology, (1990 Apr) Vol. 10, No. 4, pp. 1373-81.
Journal code: 8109087. ISSN: 0270-7306. L-ISSN: 0270-7306.
Report No.: NLM-PMC362239.
CY United States
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
OS GENBANK-M27418
EM 199005
ED Entered STN: 1 Jun 1990
Last Updated on STN: 29 Jan 1999
Entered Medline: 2 May 1990
OSC.G 9 There are 9 MEDLINE records that cite this record
REM.CNT 45 There are 45 cited references available in MEDLINE for this document.
AB There are a number of yeasts that secrete killer toxins, i.e., proteins lethal to sensitive cells of the same or related species. Ustilago maydis, a fungal pathogen of maize, also secretes killer toxins. The best characterized of the U. maydis killer toxins is the KP6 toxin, which consists of two small polypeptides that are not covalently linked. In this work, we show that both are encoded by one segment of the genome of a double-stranded RNA virus. They are synthesized as a preprotoxin that is processed in a manner very similar to that of the Saccharomyces cerevisiae k1 killer toxin, also encoded by a double-strand RNA virus. Active U. maydis KP6 toxin was secreted from S. cerevisiae transformants expressing the KP6 preprotoxin. The two secreted polypeptides were not glycosylated in U. maydis, but one was glycosylated in S. cerevisiae. Comparison of known and predicted cleavage sites among the five killer toxins of known sequence established a three-amino-acid specificity for a KEX2-like enzyme and predicted a new, undescribed processing enzyme in the secretory pathway in the fungi. The mature KP6 toxin polypeptides had hydrophobicity profiles similar to those of other known cellular toxins.

L7 ANSWER 127 OF 184 MEDLINE ® on STN DUPLICATE 101
AN 2009059604 MEDLINE Full-text
DN PubMed ID: 19130676
TI Isolation and properties of a chromosome-dependent KHR killer toxin in Saccharomyces cerevisiae.
AU Goto K; Iwase T; Kichise K; Kitano K; Totuka A; Obata T; Hara S
CS National Research Institute of Brewing, 2-6-30 Takinogawa, Kita-ku, Tokyo 114, Japan.
SO Agricultural and biological chemistry, (1990) Vol. 54, No. 2, pp. 505-9.
Journal code: 0370452. ISSN: 0002-1369. L-ISSN: 0002-1369.
CY Japan
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Biotechnology
EM 200903
ED Entered STN: 10 Jan 2009
Last Updated on STN: 6 Mar 2009
Entered Medline: 5 Mar 2009
OSC.G 2 There are 2 MEDLINE records that cite this record
AB A strain of the yeast Saccharomyces cerevisiae coding for KHR on the chromosome secreted a toxin that kills sensitive yeasts. The transformants of multicopy vectors carrying the KHR gene could secrete 3-4-fold the killer toxin of the donor strain. This toxic substance was purified 80-fold in specific activity from the culture filtrate by gel filtration and hydrophobic column chromatography. The purified toxin gave a single protein band with molecular mass of 20 kDa on SDS-PAGE and had an isoelectric point of pH 5.3. The toxin had novel killer activity against Candida glabrata and S. cerevisiae, but did not affect bacteria, fungi, or other yeasts.

L7 ANSWER 128 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1991:18687 BIOSIS Full-text
DN PREV199140007017; BR40:7017
TI EXPRESSION OF TETANUS TOXIN FRAGMENT C IN SACCHAROMYCES COMPLETE GENE SYNTHESIS IS REQUIRED TO ELIMINATE MULTIPLE TRANSCRIPTIONAL TERMINATORS.
AU ROMANOS M A [Reprint author]; MAKOFF A J; BEESLEY K M; RAYMENT F; CLARE J J
CS WELLCOME BIOTECHN, LANGLEY COURT, BECKENHAM, KENT BR3 3BS, UK
SO Yeast, (1990) Vol. 6, No. SPEC. ISSUE, pp. S427.
Meeting Info.: FIFTEENTH INTERNATIONAL CONFERENCE ON YEAST GENETICS AND MOLECULAR BIOLOGY, THE HAGUE, NETHERLANDS, JULY 21-26, 1990. YEAST.
CODEN: YESTE3. ISSN: 0749-503X.
DT Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 11 Dec 1990

Last Updated on STN: 11 Dec 1990

L7 ANSWER 129 OF 184 MEDLINE ® on STN DUPLICATE 102
AN 1990094236 MEDLINE Full-text
DN PubMed ID: 2403543
TI Cu,Zn superoxide dismutase and copper deprivation and toxicity in Saccharomyces cerevisiae.
AU Greco M A; Hrab D I; Magner W; Kosman D J
CS Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York, Buffalo 14214.
NC AM-197089 (United States NIADDK NIH HHS)
SO Journal of bacteriology, (1990 Jan) Vol. 172, No. 1, pp. 317-25.
Journal code: 2985120R. ISSN: 0021-9193. L-ISSN: 0021-9193.
Report No.: NLM-PMC208435.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 199002
ED Entered STN: 28 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 8 Feb 1990
OSC.G 4 There are 4 MEDLINE records that cite this record
REM.CNT 39 There are 39 cited references available in MEDLINE for this document.
AB A wild-type strain of the yeast Saccharomyces cerevisiae grown at a medium [Cu] of less than or equal to 50 nM contained less Cu,Zn superoxide dismutase (SOD) mRNA (60%), protein (50%), and activity (50%) in comparison with control cultures grown in normal synthetic dextrose medium ([Cu] approximately 150 nM). A compensating increase in the activity of MnSOD was observed, as well as a smaller increase in MnSOD mRNA. These medium [Cu]-dependent differences were observed in cultures under N2 as well. Addition of Cu2+ (100 microM) to Cu-depleted cultures resulted in a rapid (30 min) increase in Cu,ZnSOD mRNA (2.5-fold), protein (3.5-fold), and activity (4-fold). Ethidium bromide (200 micrograms/ml of culture) inhibited by 50% the increase in Cu,ZnSOD mRNA, while cycloheximide (100 micrograms/ml of culture) inhibited completely the increase in protein and activity. Addition of Cu2+ to greater than or equal to 100 microM caused no further increase in these parameters but did result in a loss of total cellular RNA and translatable RNA, a decline in the population of specific mRNAs, a decrease in total soluble protein and the activity of specific enzymes, and an inhibition of incorporation of [3H]uracil and [3H]leucine into trichloroacetic acid-insoluble material. Cu,ZnSOD mRNA, protein, and activity appeared relatively more resistant to these effects of Cu toxicity than did the other cellular constituents examined. When evaluated in cultures under N2, the cellular response to [Cu] of greater than or equal to 100 microM was limited to the inhibition of radiolabel incorporation into trichloroacetic acid-insoluble material. All other effects were absent in the absence of O2. The data indicated that medium (cellular) Cu alters the steady-state level of Cu, ZnSOD. This regulation may be at the level of transcription. In addition, Cu,ZnSOD exhibits the characteristics of Cu-stress protein in that it and its mRNA are enhanced relative to other cellular species under conditions of Cu excess. This observation and the O2-dependence of some of the manifestations of Cu excess suggest that one mechanism of Cu toxicity involves the superoxide radical anion O2-.

L7 ANSWER 130 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 103
AN 1989:430875 BIOSIS Full-text
DN PREV198988089133; BA88:89133
TI THE K1 TOXIN OF SACCHAROMYCES-CEREVISIAE KILLS SPHEROPLASTS OF MANY YEAST SPECIES.
AU ZHU H [Reprint author]; BUSSEY H
CS DEP BIOL, MCGILL UNIV, 1205 AVE DR PENFIELD, MONTREAL, QUEBEC, CANADA H3A 1B1
SO Applied and Environmental Microbiology, (1989) Vol. 55, No. 8, pp. 2105-2107.
CODEN: AEMIDF. ISSN: 0099-2240.
DT Article
FS BA
LA ENGLISH
ED Entered STN: 19 Sep 1989
Last Updated on STN: 19 Sep 1989
AB The Saccharomyces cerevisiae K1 toxin killed spheroplasts from the genera Candida, Kluyveromyces, and Schwanniomyces. Cells of these organisms were toxin insensitive. The toxin bound poorly to Kluyveromyces factis cells. In contrast, Candida albicans bound the toxin to an extent similar to that seen with S. cerevisiae. Thus, wall receptors can define toxin specificity and are necessary but not sufficient for toxin action on intact cells.

L7 ANSWER 131 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1989:458307 BIOSIS Full-text
DN PREV198937090951; BR37:90951
TI STRUCTURAL ANALYSIS OF BETA GLYCANS FROM A KILLER TOXIN SENSITIVE YEAST SACCHAROMYCES-CEREVISIAE AND A KILLER-RESISTANT MUTANT.
AU NAKAJIMA T [Reprint author]; AOYAMA K; ICHISHIMA E; MATSUDA K
CS DEP AGRIC CHEM, FAC AGRIC, TOHOKU UNIV, TSUTMIDORI-AMAMIYAMACHI, SENDAI 980, JPN
SO Agricultural and Biological Chemistry, (1989) Vol. 53, No. 7, pp. 1983-1986.
CODEN: ABCHA6. ISSN: 0002-1369.
DT Article
FS BR
LA ENGLISH
ED Entered STN: 12 Oct 1989
Last Updated on STN: 12 Oct 1989

L7 ANSWER 132 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 104
AN 1989:428019 BIOSIS Full-text
DN PREV198988086277; BA88:86277
TI EFFECT OF SACCHAROMYCES-BOULARDII ON CHOLERA TOXIN INDUCED CAMP LEVEL IN RAT EPITHELIAL INTESTINAL CELL LINES.
AU CZERUCKA D [Reprint author]; NANO J L; BERNASCONI P; RAMPAL P

CS LAB DE GASTROENTEROLOGIE ET NUTRITION, FAC DE MED, AVE DE VALOMBROSE, F-06034 NICE CEDEX
SO Gastroenterologie Clinique et Biologique, (1989) Vol. 13, No. 4, pp. 383-387.
CODEN: GCBIDC. ISSN: 0399-8320.
DT Article
FS BA
LA FRENCH
ED Entered STN: 19 Sep 1989
Last Updated on STN: 23 Sep 1989
AB Cholera toxin acts in vivo by activating intestinal adenylate cyclase. This study was designed to determine (1) whether normal rat epithelial intestinal cell lines (IRD 98 and IEC 17) respond to cholera toxin (CT) by an increased concentration of cyclic AMP and (2) whether the yeast Saccharomyces boulardii, which reduced CT-induced secretion of water and electrolytes using the isolated jejunal loop technique, has an effect on these models. The cAMP concentration evaluated in cells exposed to Saccharomyces boulardii and to cholera toxin (1 µg/ml for 90 min) was compared to the concentration of cAMP obtained in control cells with yeast. Prior exposure of IRD 98 and IEC 17 cells to Saccharomyces boulardii, reduced CT-induced cAMP by 50 p. 100. This effect disappeared after destruction of the yeast by heating. Results show that the IRD 98 and IEC 17 cells are good models for an in vitro investigation of the effects of cholera toxin. Our results suggests that Saccharomyces boulardii prevents the water and electrolyte secretion induced by cholera toxin.

L7 ANSWER 133 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1990:113800 BIOSIS Full-text
DN PREV199089063291; BA89:63291
TI COPPER ZINC SUPEROXIDE DISMUTASE AND COPPER DEPRIVATION AND TOXICITY IN SACCHAROMYCES-CEREVISIAE.
AU GRECO M A [Reprint author]; HRAB D I; MAGNER W; KOSMAN D J
CS DEP BIOCHEM, 140 FARBER HALL, SCH OF MED AND BIOMED SCI, STATE UNIV OF NEW YORK AT BUFFALO, BUFFALO, NEW YORK 14214, USA
SO Journal of Bacteriology, (1989) Vol. 72, No. 1, pp. 317-325.
CODEN: JOBAAY. ISSN: 0021-9193.
DT Article
FS BA
LA ENGLISH
ED Entered STN: 21 Feb 1990
Last Updated on STN: 22 Feb 1990
AB A wild-type strain of the yeast Saccharomyces cerevisiae grown at a medium [Cu] of ≤ 50 nM contained less Cu,Zn superoxide dismutase (SOD) mRNA (60%), protein (50%), and activity (50%) in comparison with control cultures grown in normal synthetic dextrose medium ([Cu] ≈ 150 nM). A compensating increase in the activity of MnSOD was observed, as well as a smaller increase in MnSOD mRNA. These medium [Cu]-dependent differences were observed in cultures under N2 as well. Addition of Cu2+ (100 µM) to Cu-depleted cultures resulted in a rapid (30 min) increase in Cu,ZnSOD mRNA (2.5-fold), protein (3.5-fold), and activity (4-fold). Ethidium bromide (200 µg/ml of culture) inhibited by 50% the increase in Cu,ZnSOD mRNA, while cycloheximide (100 µg/ml of culture) inhibited completely the increase in protein and activity. Addition of Cu2+ to ≥ 100 µM caused no further increase in these parameters but did result in a loss of total cellular RNA and translatable RNA, a decline in the population of specific mRNAs, a decrease in total soluble protein and the activity of specific enzymes, and an inhibition of incorporation of [3H]uracil and [3H]leucine into trichloroacetic acid-insoluble material. Cu,ZnSOD mRNA, protein, and activity appeared relatively more resistant to these effects of Cu toxicity than did the other cellular constituents examined. When evaluated in cultures under N2, the cellular response to [Cu] of ≥ 100 µM was limited to the inhibition of radiolabel incorporation into trichoroacetic acid-insoluble material. All other effects were absent in the absence of O2. The data indicated that medium (cellular) Cu alters the steady-state level of Cu,ZuSOD. This regulation may be at the level of transcription. In addition, Cu,ZnSOD exhibits the characteristics of a Cu-stress protein in that it and its mRNA are enhanced relative to other cellular species under conditions of Cu excess. This observation and the O2-dependence of some of the manifestations of Cu excess suggest that one mechanism of Cu toxicity involves the superoxide radical anion, O2-.

L7 ANSWER 134 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1989:497351 BIOSIS Full-text
DN PREV198988123888; BA88:123888
TI INFLUENCE OF HEXACHLOROCYCLOHEXANE ISOMERS ON SACCHAROMYCES-CEREVISIAE ACCUMULATION AND TOXICITY STUDIES.
AU MATHUR R [Reprint author]; SAXENA D M
CS DEP ZOOL, UNIV DELHI, DELHI-110 007
SO Journal of Environmental Biology, (1989) Vol. 10, No. 2 SUPPL, pp. 227-236.
CODEN: JEBIDP. ISSN: 0254-8704.
DT Article
FS BA
LA ENGLISH
ED Entered STN: 2 Nov 1989
Last Updated on STN: 4 Nov 1989
AB The effect of four common isomers of HCH viz., α, β, δ and γ on the growth of the yeast, Saccharomyces cerevisae was investigated. At concentrations of 1 and 10 ppm, the γ-isomer inhibited the growth of the yeast more than other isomers but at 50 and 100 ppm, β-HCH was the most inhibitory isomer. Saccharomyces accumulated the HCH isomers from the medium. The bioconcentration ratio (the concentration of the organism/concentration in the medium) taking into consideration the maximum amount of the isomer accumlated followed the sequence γ = δ > β > α

L7 ANSWER 135 OF 184 MEDLINE ® on STN DUPLICATE 105
AN 1988198010 MEDLINE Full-text
DN PubMed ID: 2834332
TI Molecular structure of the cell wall receptor for killer toxin KT28 in Saccharomyces cerevisiae.
AU Schmitt M; Radler F
CS Institut fur Mikrobiologie und Weinforschung, Johannes Gutenberg-Universitat Mainz, Federal Republic of Germany.
SO Journal of bacteriology, (1988 May) Vol. 170, No. 5, pp. 2192-6.
Journal code: 2985120R. ISSN: 0021-9193. L-ISSN: 0021-9193.
Report No.: NLM-PMC211105.
CY United States

DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198806
ED Entered STN: 8 Mar 1990
Last Updated on STN: 8 Mar 1990
Entered Medline: 9 Jun 1988
OSC.G 7 There are 7 MEDLINE records that cite this record
REM.CNT 14 There are 14 cited references available in MEDLINE for this document.
AB The adsorption of the yeast killer toxin KT28 to susceptible cells of Saccharomyces cerevisiae was prevented by concanavalin A, which blocks the mannoprotein receptor. Certain mannoprotein mutants of S. cerevisiae that lack definite structures in the mannan of their cell walls were found to be resistant to KT28, whereas the wild-type yeast from which the mutants were derived was susceptible. Isolated mannoprotein from a resistant mutant was unable to adsorb killer toxin. By comparing the resistances of different mannoprotein mutants, information about the molecular structure of the receptor was obtained. At least two mannose residues have to be present in the side chains of the outer chain of the cell wall mannan, whereas the phosphodiester-linked mannose group is not essential for binding and the subsequent action of killer toxin KT28.

L7 ANSWER 136 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1988:345812 BIOSIS Full-text
DN PREV198835040654; BR35:40654
TI RESPONSE OF TWO EPITHELIAL INTESTINAL CELL LINES TO CHOLERA TOXIN EFFECT OF SACCHAROMYCES-BOULARDII.
AU CZERUCKA D [Reprint author]; NANO J L; BERNASCONI P; RAMPAL P
CS LAB DE GASTRO-ENTEROL, UER DE MED, 06034 NICE CEDEX, FR
SO Gastroenterology, (1988) Vol. 94, No. 5 PART 2, pp. A82.
Meeting Info.: 89TH ANNUAL MEETING OF THE AMERICAN GASTROENTEROLOGICAL ASSOCIATION, NEW ORLEANS, LOUISIANA, USA, MAY 14-20, 1988.
GASTROENTEROLOGY.
CODEN: GASTAB. ISSN: 0016-5085.
DT Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 26 Jul 1988
Last Updated on STN: 26 Jul 1988

L7 ANSWER 137 OF 184 MEDLINE ® on STN DUPLICATE 106
AN 1989036079 MEDLINE Full-text
DN PubMed ID: 3332684
TI Mannoprotein of the yeast cell wall as primary receptor for the killer toxin of Saccharomyces cerevisiae strain 28.
AU Schmitt M; Radler F
CS Institut fur Mikrobiologie und Weinforschung, Johannes Gutenberg-Universitat Mainz, FRG.
SO Journal of general microbiology, (1987 Dec) Vol. 133, No. 12, pp. 3347-54.
Journal code: 0375371. ISSN: 0022-1287. L-ISSN: 0022-1287.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198812
ED Entered STN: 8 Mar 1990
Last Updated on STN: 8 Mar 1990
Entered Medline: 12 Dec 1988
OSC.G 11 There are 11 MEDLINE records that cite this record
AB The killer toxin KT 28 of Saccharomyces cerevisiae strain 28 is primarily bound to the mannoprotein of the cell wall of sensitive yeasts. The mannoprotein of S. cerevisiae X 2180 was purified; gel filtration and SDS-PAGE indicated an estimated Mr of 185,000. The ability to bind killer toxin KT 28 increased during purification of the mannoprotein. Removing the protein part of the mannoprotein by enzymic digestion or removing the alkali-labile oligosaccharide chains by beta-elimination did not destroy the ability to bind killer toxin KT 28. However, binding activity was lost when the 1,6-alpha-linkages of the outer carbohydrate backbone were hydrolysed by acetolysis. The separated oligomannosides of the side chains also failed to bind toxin, indicating that the main mannoside chains were essential for the receptor activity. The reversible adsorption of killer toxin to mannoprotein was demonstrated by linking it covalently to Sepharose and using this material for affinity chromatography. A 90-fold increase in the specific activity of a preparation of killer toxin KT 28 was achieved in this way.

L7 ANSWER 138 OF 184 MEDLINE ® on STN DUPLICATE 107
AN 1987304754 MEDLINE Full-text
DN PubMed ID: 3305064
TI Protective effect of vitamins against trichothecene toxicity towards Saccharomyces cerevisiae.
AU Yagen B; Halevy S
SO Experientia, (1987 Aug 15) Vol. 43, No. 8, pp. 886-8.
Journal code: 0376547. ISSN: 0014-4754. L-ISSN: 0014-4754.
CY Switzerland
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198709
ED Entered STN: 5 Mar 1990
Last Updated on STN: 5 Mar 1990
Entered Medline: 30 Sep 1987
AB Several trichothecene mycotoxins were shown to inhibit the growth of Saccharomyces cerevisiae. This effect was most pronounced with the macrocyclic trichothecenes, especially verrucarin A. Much less growth inhibition was observed with T-2 toxin. Verrucarol, diacetoxyscirpenol, acetyl T-2 toxin, HT-2 toxin, T-2 tetraol and neosolaniol were inactive at a concentration of 75 micrograms of toxin per disc. Incubation of S. cerevisiae with verrucarin A together with vitamins resulted in a decrease in toxicity. Pyridoxine-HCl, Ca-pantothenate, thiamine-HCl and alpha-tocopheryl acetate were amongst the most potent of the vitamins tested which reversed growth inhibition, overcoming the inhibitory potential of the toxins.

L7 ANSWER 139 OF 184 MEDLINE ® on STN DUPLICATE 108
AN 1987193402 MEDLINE Full-text
DN PubMed ID: 3566236
TI Suppression by Saccharomyces boulardii of toxigenic Clostridium difficile overgrowth after vancomycin treatment in hamsters.
AU Elmer G W; McFarland L V
SO Antimicrobial agents and chemotherapy, (1987 Jan) Vol. 31, No. 1, pp. 129-31.
Journal code: 0315061. ISSN: 0066-4804. L-ISSN: 0066-4804.
Report No.: NLM-PMC174670.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198704
ED Entered STN: 3 Mar 1990
Last Updated on STN: 6 Feb 1995
Entered Medline: 29 Apr 1987
OSC.G 7 There are 7 MEDLINE records that cite this record
REM.CNT 20 There are 20 cited references available in MEDLINE for this document.
AB Saccharomyces boulardii prevented the development of high counts of Clostridium difficile, high titers of toxin B, and positive latex agglutination tests after cessation of vancomycin treatment for hamsters. The protocol used was designed to stimulate relapse of human C. difficile-associated colitis. S. boulardii was protective in this model.

L7 ANSWER 140 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1986:464282 BIOSIS Full-text
DN PREV198631111290; BR31:111290
TI MATURATION AND SECRETION OF THE M-1 DOUBLE-STRANDED RNA ENCODED KILLER TOXIN IN SACCHAROMYCES-CEREVISIAE.
AU STURLEY S L [Reprint author]; HANES S D; BURN V; BOSTIAN K A
CS DIV BIOLOGY AND MED, SECTION BIOCHEMISTRY, BROWN UNIV, PROVIDENCE, RHODE ISLAND 02912, USA
SO UCLA Symp. Mol. Cell. Biol., New Ser., (1986) pp. 537-550. HICKS, J. (ED.). UCLA (UNIVERSITY OF CALIFORNIA-LOS ANGELES) SYMPOSIA ON MOLECULAR AND CELLULAR BIOLOGY NEW SERIES, VOL. 33. YEAST CELL BIOLOGY; PROCEEDINGS OF A CETUS-UCLA SYMPOSIUM, KEYSTONE, COLO., USA, APR. 9-15, 1985. XXI+671P. ALAN R. LISS, INC.: NEW YORK, N.Y., USA. ILLUS.
Publisher: Series: UCLA (University of California Los Angeles) Symposia on Molecular and Cellular Biology New Series.
CODEN: USMBD6. ISSN: 0735-9543. ISBN: 0-8451-2632-6.
DT Book
Conference; (Meeting)
FS BR
LA ENGLISH
ED Entered STN: 22 Nov 1986
Last Updated on STN: 22 Nov 1986

L7 ANSWER 141 OF 184 MEDLINE ® on STN DUPLICATE 109
AN 1986310812 MEDLINE Full-text
DN PubMed ID: 3915773
TI Diphtheria toxin-resistant mutants of Saccharomyces cerevisiae.
AU Chen J Y; Bodley J W; Livingston D M
NC GM-26832 (United States NIGMS NIH HHS)
SO Molecular and cellular biology, (1985 Dec) Vol. 5, No. 12, pp. 3357-60.
Journal code: 8109087. ISSN: 0270-7306. L-ISSN: 0270-7306.
Report No.: NLM-PMC369163.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 198610
ED Entered STN: 21 Mar 1990
Last Updated on STN: 3 Mar 2000
Entered Medline: 3 Oct 1986
OSC.G 8 There are 8 MEDLINE records that cite this record
REM.CNT 26 There are 26 cited references available in MEDLINE for this document.
AB We developed a selection procedure based on the observation that diphtheria toxin kills spheroplasts of Saccharomyces cerevisiae (Murakami et al., Mol. Cell. Biol. 2:588-592, 1982); this procedure yielded mutants resistant to the in vitro action of the toxin. Spheroplasts of mutagenized S. cerevisiae were transformed in the presence of diphtheria toxin, and the transformed survivors were screened in vitro for toxin-resistant elongation factor 2. Thirty-one haploid ADP ribosylation-negative mutants comprising five complementation groups were obtained by this procedure. The mutants grew normally and were stable to prolonged storage. Heterozygous diploids produced by mating wild-type sensitive cells with the mutants revealed that in each case the resistant phenotype was recessive to the sensitive phenotype. Sporulation of these diploids yielded tetrads in which the resistant phenotype segregated as a single Mendelian character. From these observations, we concluded that these mutants are defective in the enzymatic steps responsible for the posttranslational modification of elongation factor 2 which is necessary for recognition by diphtheria toxin.

L7 ANSWER 142 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 110
AN 1986:117066 BIOSIS Full-text
DN PREV198681027482; BA81:27482
TI PROTECTION OF SACCHAROMYCES-CEREVISIAE AGAINST CADMIUM TOXICITY BY CALCIUM.
AU KESSELS B G F [Reprint author]; BELDE P J M; BORST-PAUWELS G W F H
CS DEP CHEM CYTOL, FAC SCI, UNIV NIJMEGEN, TOERNOOIVELD, 6525 ED NIJMEGEN,

NETH
SO Journal of General Microbiology, (1985) Vol. 131, No. 10, pp. 2533-2538.
CODEN: JGMIAN. ISSN: 0022-1287.
DT Article
FS BA
LA ENGLISH
ED Entered STN: 25 Apr 1986
Last Updated on STN: 25 Apr 1986
AB Ca2+ protected yeast cells very effectively against the toxic effects of Cd2+; Mg2+ had only a slight protecting effect as far as protection against Cd2+-induced release of K+ was concerned. Protection of the yeast cells against Cd2+ toxicity was due to a reduction in Cd2+ uptake in the presence of Ca2+. A single relationship existed between the relative rate of K+ release induced by Cd2+ and the cellular Cd2+ concentration. Within the first few minutes of incubating cells with Cd2+, the molar ratio of K+ released and Cd2+ accumulated was 22 and was independent of the amount of CdCl2 added. This ratio decreased during incubation of the cells with Cd2+, depending on the external Cd2+ concentration.

L7 ANSWER 143 OF 184 MEDLINE ® on STN DUPLICATE 111
AN 1986083151 MEDLINE Full-text
DN PubMed ID: 4076767
TI [Comparative genetics of yeasts. XXIII. Unusual inheritance of toxin formation in Saccharomyces paradoxus batschinskaia].
Sravnitel'naia genetika drozhzhei. Soobshchenie XXIII. Neobychainoe nasledovanie toksinoobrazovaniia u Saccharomyces paradoxus batschinskaia.
AU Naumov G I
SO Genetika, (1985 Nov) Vol. 21, No. 11, pp. 1794-8.
Journal code: 0047354. ISSN: 0016-6758. L-ISSN: 0016-6758.
CY USSR
DT (COMPARATIVE STUDY)
(ENGLISH ABSTRACT)
Journal; Article; (JOURNAL ARTICLE)
LA Russian
FS MEDLINE; Priority Journals
EM 198602
ED Entered STN: 21 Mar 1990
Last Updated on STN: 21 Mar 1990
Entered Medline: 19 Feb 1986
AB The data about cytoplasmic control of toxin formation in Saccharomyces paradoxus CBS5829 are presented. A novel determinant (KIL-k3) is probably located in the mitochondrial genome. In other mutations, adenine deficiency results in suppression of toxin formation of the K3 type. A new killer plasmid (KIL-kx) was detected in Sacch. paradoxus VKM Y-2472.

L7 ANSWER 144 OF 184 MEDLINE ® on STN DUPLICATE 112
AN 1986024219 MEDLINE Full-text
DN PubMed ID: 4051481
TI Phenotypic expression of Kluyveromyces lactis killer toxin against Saccharomyces spp.
AU Panchal C J; Meacher C; Van Oostrom J; Stewart G G
SO Applied and environmental microbiology, (1985 Aug) Vol. 50, No. 2, pp. 257-60.
Journal code: 7605801. ISSN: 0099-2240. L-ISSN: 0099-2240.
Report No.: NLM-PMC238612.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 198510
ED Entered STN: 21 Mar 1990
Last Updated on STN: 29 Jan 1999
Entered Medline: 30 Oct 1985
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 18 There are 18 cited references available in MEDLINE for this document.
AB The secretion of killer toxins by some strains of yeasts is a phenomenon of significant industrial importance. The activity of a recently discovered Kluyveromyces lactis killer strain against a sensitive Saccharomyces cerevisiae strain was determined on peptone-yeast extract-nutrient agar plates containing as the carbon source glucose, fructose, galactose, maltose, or glycerol at pH 4.5 or 6.5. Enhanced activity (50 to 90% increase) was found at pH 6.5, particularly on the plates containing galactose, maltose, or glycerol, although production of the toxin in liquid medium was not significantly different with either glucose or galactose as the carbon source. Results indicated that the action of the K. lactis toxin was not mediated by catabolite repression in the sensitive strain. Sensitivities of different haploid and polyploid Saccharomyces yeasts to the two different killer yeasts S. cerevisiae (RNA-plasmid-coded toxin) and K. lactis (DNA-plasmid-coded toxin) were tested. Three industrial polyploid yeasts sensitive to the S. cerevisiae killer yeast were resistant to the K. lactis killer yeast. The S. cerevisiae killer strain itself, however, was sensitive to the K. lactis killer yeast.

L7 ANSWER 145 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1985:152661 BIOSIS Full-text
DN PREV198529042657; BR29:42657
TI THE RELATIONSHIP OF METHYLAMINE TRANSPORT TO NITROGEN CATABOLITE REPRESSION AND TO METHYLAMINE TOXICITY IN SACCHAROMYCES-CEREVISIAE.
AU ALEXANDER P [Reprint author]; ADAMS B G
CS UNIV HAWAII, MANOA, HONOLULU, HAWAII 96822, USA
SO Abstracts of the Annual Meeting of the American Society for Microbiology, (1985) Vol. 85, pp. 191.
Meeting Info.: 85TH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, LAS VEGAS, NEV., USA, MAR. 3-7, 1985. ABSTR ANNU MEET AM SOC MICROBIOL.
CODEN: ASMACK. ISSN: 0094-8519.
DT Conference; (Meeting)
FS BR
LA ENGLISH

L7 ANSWER 146 OF 184 MEDLINE ® on STN DUPLICATE 113
AN 1985036325 MEDLINE Full-text
DN PubMed ID: 6387446
TI Double-stranded RNAs that encode killer toxins in Saccharomyces cerevisiae: unstable size of M double-stranded RNA and inhibition of M2 replication by M1.
AU Sommer S S; Wickner R B
SO Molecular and cellular biology, (1984 Sep) Vol. 4, No. 9, pp. 1747-53.
Journal code: 8109087. ISSN: 0270-7306. L-ISSN: 0270-7306.
Report No.: NLM-PMC368982.
CY United States
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198412
ED Entered STN: 20 Mar 1990
Last Updated on STN: 20 Mar 1990
Entered Medline: 12 Dec 1984
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 35 There are 35 cited references available in MEDLINE for this document.
AB The sizes of M1 and M2 (but not L) change rapidly with growth, varying by perhaps as much as 33%. Size variation is seen within 76 generations. In addition, the exclusion of M2 by M1 or L-A-E [(EXL]) is mediated by inhibition of replication or segregation, not by enhanced degradation of preexisting molecules.

L7 ANSWER 147 OF 184 MEDLINE ® on STN DUPLICATE 114
AN 1984201773 MEDLINE Full-text
DN PubMed ID: 6372690
TI Influence of the membrane on T-2 toxin toxicity in Saccharomyces spp.
AU Schappert K T; Khachatourians G G
SO Applied and environmental microbiology, (1984 Apr) Vol. 47, No. 4, pp. 681-4.
Journal code: 7605801. ISSN: 0099-2240. L-ISSN: 0099-2240.
Report No.: NLM-PMC239748.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 198406
ED Entered STN: 19 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 20 Jun 1984
OSC.G 4 There are 4 MEDLINE records that cite this record
REM.CNT 17 There are 17 cited references available in MEDLINE for this document.
AB In growing cells of Saccharomyces cerevisiae and Saccharomyces carlsbergensis, T-2 toxin inhibits cell growth. We have examined the role of the yeast membranes in the uptake mechanism(s) of T-2 toxin. The effects of membrane-modulating agents, ethanol, cetyltrimethylammonium bromide, Triton X-100, and heat were studied; these agents were found to increase the sensitivity of the yeasts toward T-2 toxin. In the presence of 5% (vol/vol) ethanol, 2 micrograms of T-2 toxin per ml caused complete inhibition of growth. In the presence of 1 microgram of cetyltrimethylammonium bromide per ml, yeast cells became sensitive to T-2 toxin, starting with a concentration of 0.5 micrograms/ml. Triton X-100 at concentrations below 1% (vol/vol) sensitized the cells toward T-2 toxin, but at higher concentrations it protected the cells from T-2 toxin. Temperatures of incubation between 7 and 30 degrees C influenced the growth reduction caused by T-2 toxin. The greatest observed reduction of growth in T-2 toxin-treated cultures occurred at 30 degrees C. To further prove that the membrane influences the interaction of T-2 toxin with yeasts, we have studied a yeast mutant with a reduced plasma membrane permeability (G. H. Rank et al., Mol. Gen. Genet. 152:13-18, 1977). This yeast mutant proved to be resistant to T-2 toxin concentrations of up to 50 micrograms/ml. These results show that the membrane plays a significant role in the interaction of T-2 toxin with yeast cells.

L7 ANSWER 148 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1984:340744 BIOSIS Full-text
DN PREV198478077224; BA78:77224
TI MECHANISMS OF CISPLATIN CIS DI AMMINE DI CHLORO PLATINUM II INDUCED CYTO TOXICITY AND GENO TOXICITY IN YEAST SACCHAROMYCES-CEREVISIAE.
AU HANNAN M A [Reprint author]; ZIMMER S G; HAZLE J
CS DEPARTMENT OF CANCER AND RADIATION BIOLOGY, CANCER THERAPY INSTITUTE, KING FAISAL SPECIALIST HOSPITAL AND RESEARCH CENTRE, PO BOX 3354, RIYADH 11211, SAUDI ARABIA
SO Mutation Research, (1984) Vol. 127, No. 1, pp. 23-30.
CODEN: MUREAV. ISSN: 0027-5107.
DT Article
FS BA
LA ENGLISH
AB The antitumor drug, cisplatin (cis-diamminodichloroplatinum II), dissolved in both water and phosphate-buffered saline, was studied for its genotoxic and cytotoxic effects in the yeast, S. cerevisiae. The drug was both recombinagenic and mutagenic in the wild-type diploid strain D7. Both cytotoxicity and genotoxicity were greatly reduced when cisplatin was dissolved in phosphate-buffered saline compared to the aqueous solution. Cell survival analyses showed that the diploid strain (D7 rad 3), deficient in excision of UV-induced pyrimidine dimers or similar adducts, was hypersensitive to cisplatin. Another diploid strain (rad 52/rad 52), blocked in the repair of DNA double-strand breaks and recombination was also hypersensitive to the drug. Mitotic gene conversion was not observed in the rad 52/rad 52 diploid after the drug treatments, while it was reduced in the excision-deficient strain. Reverse mutations occurred in the excision-deficient strain (D7 rad 3), even at low doses of cisplatin. The possible mechanisms of cisplatin-induced cell death and genotoxicity are discussed.

L7 ANSWER 149 OF 184 MEDLINE ® on STN DUPLICATE 115
AN 1984013757 MEDLINE Full-text
DN PubMed ID: 6353202
TI Secretion of Saccharomyces cerevisiae killer toxin: processing of the

glycosylated precursor.
AU Bussey H; Saville D; Greene D; Tipper D J; Bostian K A
NC GM20755 (United States NIGMS NIH HHS)
SO Molecular and cellular biology, (1983 Aug) Vol. 3, No. 8, pp. 1362-70.
Journal code: 8109087. ISSN: 0270-7306. L-ISSN: 0270-7306.
Report No.: NLM-PMC369982.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, P.H.S.)
LA English
FS MEDLINE; Priority Journals
EM 198311
ED Entered STN: 19 Mar 1990
Last Updated on STN: 3 Feb 1997
Entered Medline: 23 Nov 1983
OSC.G 15 There are 15 MEDLINE records that cite this record
REM.CNT 18 There are 18 cited references available in MEDLINE for this document.
AB Killer toxin secretion was blocked at the restrictive temperature in Saccharomyces cerevisiae sec mutants with conditional defects in the S. cerevisiae secretory pathway leading to accumulation of endoplasmic reticulum (sec18), Golgi (sec7), or secretory vesicles (sec1). A 43,000-molecular-weight (43K) glycosylated protoxin was found by pulse-labeling in all sec mutants at the restrictive temperature. In sec18 the protoxin was stable after a chase; but in sec7 and sec1 the protoxin was unstable, and in sec1 11K toxin was detected in cell lysates. The chymotrypsin inhibitor tosyl-l-phenylalanyl chloromethyl ketone (TPCK) blocked toxin secretion in vivo in wild-type cells by inhibiting protoxin cleavage. The unstable protoxin in wild-type and in sec7 and sec1 cells at the restrictive temperature was stabilized by TPCK, suggesting that the protoxin cleavage was post-sec18 and was mediated by a TPCK-inhibitable protease. Protoxin glycosylation was inhibited by tunicamycin, and a 36K protoxin was detected in inhibited cells. This 36K protoxin was processed, but toxin secretion was reduced 10-fold. We examined two kex mutants defective in toxin secretion; both synthesized a 43K protoxin, which was stable in kex1 but unstable in kex2. Protoxin stability in kex1 kex2 double mutants indicated the order kex1 --> kex2 in the protoxin processing pathway. TPCK did not block protoxin instability in kex2 mutants. This suggested that the KEX1- and KEX2-dependent steps preceded the sec7 Golgi block. We attempted to localize the protoxin in S. cerevisiae cells. Use of an in vitro rabbit reticulocyte-dog pancreas microsomal membrane system indicated that protoxin synthesized in vitro could be inserted into and glycosylated by the microsomal membranes. This membrane-associated protoxin was protected from trypsin proteolysis. Pulse-chased cells or spheroplasts, with or without TPCK, failed to secrete protoxin. The protoxin may not be secreted into the lumen of the endoplasmic reticulum, but may remain membrane associated and may require endoproteolytic cleavage for toxin secretion.

L7 ANSWER 150 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1983:294746 BIOSIS Full-text
DN PREV198376052238; BA76:52238
TI EFFECTS OF FUSARIO TOXIN T-2 ON SACCHAROMYCES-CEREVISIAE AND SACCHAROMYCES-CARLSBERGENSIS.
AU SCHAPPERT K T [Reprint author]; KHACHATOURIANS G G
CS DEP APPLIED MICROBIOL FOOD SCI, COLL AGRIC, UNIV SASK, SASKATOON, SASK, CAN S7N 0W0
SO Applied and Environmental Microbiology, (1983) Vol. 45, No. 3, pp. 862-867.
CODEN: AEMIDF. ISSN: 0099-2240.
DT Article
FS BA
LA ENGLISH
AB A Fusarium metabolite, T-2 toxin, inhibits the growth of S. carlsbergensis and S. cerevisiae. The growth inhibitory concentrations of T-2 toxin were 40 and 100 µg/ml, respectively, for exponentially growing cultures of the 2 yeasts. S. carlsbergensis was more sensitive to the toxin and exhibited a biphasic dose-response curve. Addition of the toxin at 10 µg/ml of S. carlsbergensis culture resulted in a retardation of growth as measured turbidimetrically, after only 30-40 min. This action was reversible upon washing the cells free of the toxin. The sensitivity of the yeasts to the toxin was dependent upon the types and concentrations of carbohydrates used in the growth media. The sensitivity of the cells to the toxin decreased in glucose-repressed cultures. These results suggest that T-2 toxin interferes with mitochondrial functions of these yeasts.

L7 ANSWER 151 OF 184 MEDLINE ® on STN DUPLICATE 116
AN 1984053448 MEDLINE Full-text
DN PubMed ID: 6315081
TI Potentiation of oxygen toxicity by menadione in Saccharomyces cerevisiae.
AU Chaput M; Brygier J; Lion Y; Sels A
SO Biochimie, (1983 Aug-Sep) Vol. 65, No. 8-9, pp. 501-12.
Journal code: 1264604. ISSN: 0300-9084. L-ISSN: 0300-9084.
CY France
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 198401
ED Entered STN: 19 Mar 1990
Last Updated on STN: 19 Mar 1990
Entered Medline: 7 Jan 1984
OSC.G 3 There are 3 MEDLINE records that cite this record
AB The cytotoxicity of molecular oxygen can be sharply increased in the yeast Saccharomyces cerevisiae by the use of redox compounds capable of shunting electrons in vivo and of spontaneous reoxidation under aerobic conditions. Among these redox compounds, menadione (Vitamin K3) is particularly able to stimulate the cyanide-resistant respiration of the yeast cells. Under steady-state conditions, the efficiency of menadione is modulated by the physiological state of the yeast cells and also depends on the availability of reducing agents within the cell. Menadione shows lethal effects towards yeast cells in the presence of O2 only, as a result of the production of toxic metabolites like O2-. and H2O2 which are actually detected in the extracellular fluid. Inhibitors of the enzymes scavenging O2-. and H2O2 generally potentiate the lethal effects of this redox compound. On the other hand, superoxide dismutase and/or catalase supplemented into the incubation buffer have been found to protect the cells to various extents from the cytotoxic effects of menadione. Our data support the following conclusions: When the cellular enzymatic defences are functional, the moderate lethality induced by menadione is principally mediated by O2-. ions acting on the outer side of the cell (peripheral region). In the presence of cyanide, but not of azide, the loss of viability also results from additional damage occurring within the inner cell region. In this case, intracellular injury can be caused by H2O2

alone but our data also suggest that during redox cycling more reactive species--O2-. and probably OH.--are generally intracellularly and are involved in the cytotoxic process.

L7 ANSWER 152 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 1984074847 EMBASE Full-text
TI Protein secretion in yeast: Two chromosomal mutants that oversecrete killer toxin in Saccharomyces cerevisiae.
AU Bussey, H.; Steinmetz, O.; Saville, D.
CS Department of Biology, McGill University, Montreal, Que. H3A 1B1, Canada.
SO Current Genetics, (1983) Vol. 7, No. 6, pp. 449-456.
ISSN: 0172-8083 CODEN: CUGED5
CY Germany
DT Journal
FS 022 Human Genetics
004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
LA English
ED Entered STN: 10 Dec 1991
Last Updated on STN: 10 Dec 1991

L7 ANSWER 153 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 1984028998 EMBASE Full-text
TI [Determination of genetic toxicity of chemicals on Saccharomyces cerevisiae. Utilization of this system in a test battery]. MESURE DE LA TOXICITE GENETIQUE DES SUBSTANCES CHIMIQUES CHEZ SACCHAROMYCES CEREVISIAE. UTILISATION DE CE SYSTEME DANS UNE BATTERIE DE TESTS.
AU Hendrickx, B.; Hermans, B.; Ottogali, M.; Lakhanisky, T.
CS Unite de Toxicologie, Institut d'Hygiene et d'Epidemiologie, B-1050 Bruxelles, Belgium.
SO Archives Belges de Medecine Sociale Hygiene Medecine du Travail et Medecine Legale, (1983) Vol. 41, No. 1-2, pp. 41-54.
ISSN: 0003-9578 CODEN: ABMHAM
CY Belgium
DT Journal
FS 017 Public Health, Social Medicine and Epidemiology
035 Occupational Health and Industrial Medicine
004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
052 Toxicology
LA French
SL English; Dutch; Flemish
ED Entered STN: 10 Dec 1991
Last Updated on STN: 10 Dec 1991

L7 ANSWER 154 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN DUPLICATE 117
AN 1983:240892 BIOSIS Full-text
DN PREV198375090892; BA75:90892
TI PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR AND INTRA CELLULAR KILLER TOXIN OF SACCHAROMYCES-CEREVISIAE STRAIN 28.
AU PFEIFFER F [Reprint author]; RADLER F
CS INSTITUT FUER MIKROBIOLOGIE UND WEINFORSCHUNG DER JOHANNES GUTENBER-UNIVERSITAET MAINZ, POSTFACH, 3980, D-6500 MAINZ, FEDERAL REPUBLIC OF GERMANY
SO Journal of General Microbiology, (1982) Vol. 128, No. 11, pp. 2699-2706.
CODEN: JGMIAN. ISSN: 0022-1287.
DT Article
FS BA
LA ENGLISH
AB The extracellular killer toxin of S. cerevisiae strain 28 was concentrated by ultrafiltration of culture supernatants and purified by ion-exchange chromatography. Polyacrylamide gradient gel electrophoresis in SDS [sodium dodecyl sulfate] indicated that the toxin is a glycoprotein with a MW of .apprx. 16,000. Amino acid analysis revealed that the killer toxin contains 111 amino acid residues, equivalent to a MW of 14,045; the ratio of protein to carbohydrate in the molecule is .apprx. 9 to 1. The isoelectric point of the killer toxin was pH 4.4-4.5. The toxin was unaffected by heating at 40° C for 1 h and its maximum activity against sensitive yeast cells occurred at pH 5.0. Cell-free extracts prepared from well-washed cells of S. cervisiae strain 28 were toxic for sensitive yeasts. The toxin present in these extracts (intracellular toxin) was partially purified by ultrafiltration and ion-exchange chromatography. The isoelectric points of the extracellular and intracellular killer toxin were similar.

L7 ANSWER 155 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 1984011609 EMBASE Full-text
TI Potentiation of oxygen toxicity by menadione in Saccharomyces cerevisiae.
AU Chaput, M.; Brygier, J.; Lion, Y.; Sels, A.
CS Dep. Biol. Mol., Univ. Libre Bruxelles, B-1640 Rhode St-Genese, Belgium.
SO Biochimie, (1982) Vol. 65, No. 8-9, pp. 501-512.
ISSN: 0300-9084 CODEN: BICMBE
CY France
DT Journal; Article
FS 037 Drug Literature Index
004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
052 Toxicology
LA English
SL French
ED Entered STN: 10 Dec 1991
Last Updated on STN: 10 Dec 1991

L7 ANSWER 156 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1983:260765 BIOSIS Full-text
DN PREV198376018257; BA76:18257
TI TESTING OF ENDOSULFAN AND FENITROTHION FOR GENO TOXICITY IN

SACCHAROMYCES-CEREVISIAE.
AU YADAV A S [Reprint author]; KAKAR S N; VASHISHAT R K
CS DEP GENET, HARYANA AGRIC UNIV, HISSAR-125004, INDIA
SO Mutation Research, (1982) Vol. 105, No. 6, pp. 403-408.
CODEN: MUREAV. ISSN: 0027-5107.
DT Article
FS BA
LA ENGLISH
AB Two insecticides, endosulfan and fenitrothion, were tested for their ability to induce mitotic crossing-over, mitotic gene conversion and reverse mutation in S. cerevisiae. Treatment of cells with endosulfan increased the frequencies of gene convertants and revertants. Fenitrothion treatment did not induce any of these genetic events. [This study may be applicable to humans.].

L7 ANSWER 157 OF 184 MEDLINE ® on STN DUPLICATE 118
AN 1982125056 MEDLINE Full-text
DN PubMed ID: 7035891
TI Mitochondrial toxicity in Saccharomyces as a measure of carcinogenicity.
AU Patel R; Wilkie D
SO Mutation research, (1982 Jan-Feb) Vol. 100, No. 1-4, pp. 179-83.
Journal code: 0400763. ISSN: 0027-5107. L-ISSN: 0027-5107.
CY Netherlands
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 198204
ED Entered STN: 17 Mar 1990
Last Updated on STN: 17 Mar 1990
Entered Medline: 12 Apr 1982

L7 ANSWER 158 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 1983070606 EMBASE Full-text
TI Genetic toxicology studies using Saccharomyces cerevisiae.
AU Zimmermann, F.K.; Mayer, V.W.; Parry, J.M.
CS Techn. Hochsch., D-6100 Darmstadt, Germany.
SO Journal of Applied Toxicology, (1982) Vol. 2, No. 1, pp. 1-10.
ISSN: 0260-437X CODEN: JJATDK
CY United States
DT Journal
FS 022 Human Genetics
004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
052 Toxicology
LA English
ED Entered STN: 9 Dec 1991
Last Updated on STN: 9 Dec 1991
AB The use of the yeast Saccharomyces cerevisiae in genetic toxicology testing is discussed. The biology of this unicellular eukaryote allows the study of genetic effects in haploid and diploid cells, and in mitotic as well as meiotic cultures. It is at present a prime organism for the study of eukaryotic molecular genetics and cell biology. The genetic system has been thoroughly analysed, and many different types of genetic end points can be studied by using genetically well-defined but simple tests. Induction of point mutation has been investigated in a number of strains, both in forward and selective reverse mutation assays. One of the most frequently studied genetic effects has been the induction of mitotic recombination. This test has shown a good response to many mutagens and carcinogens, and must be considered as a repair test since mitotic recombination is inducible by damage inflicted upon the genetic nuclear material. There are basically two types of mitotic recombination. At the intragenic level, recombination between different mutational alterations within the same gene can conveniently be studied by using selective techniques; it is due principally to the occurrence of non-reciprocal recombination between the two mutation sites and is now called mitotic gene conversion. Another type of mitotic recombination is the classical type of reciprocal crossing-over. Even though testing by using mitotic crossing-over and associated processes (cumulatively called mitotic segregation) has been based on non-selective techniques, there is a considerable volume of testing data. Tests for chromosomal malsegregation leading to aneuploidy are now available, but the number of agents tested is still small. Various important and critical aspects of using different yeast systems are discussed. Metabolic activation in yeast is provided by endogeneous P450 systems, but exogenous activation of various kinds, including that available in host mediated assays, has successfully been used.

L7 ANSWER 159 OF 184 MEDLINE ® on STN DUPLICATE 119
AN 1982048243 MEDLINE Full-text
DN PubMed ID: 7028231
TI Radioimmunoassay for yeast killer toxin from Saccharomyces cerevisiae.
AU Siddiqui F A; Bussey H
SO Canadian journal of microbiology, (1981 Aug) Vol. 27, No. 8, pp. 847-49.
Journal code: 0372707. ISSN: 0008-4166. L-ISSN: 0008-4166.
CY Canada
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 198201
ED Entered STN: 16 Mar 1990
Last Updated on STN: 16 Mar 1990
Entered Medline: 28 Jan 1982
AB A radioimmunoassay was developed for the K1 killer toxin from strain T158C/S14a of Saccharomyces cerevisiae. 125I-labeled toxin was made to a specific activity of 100 microCi/mg of protein (1 microCi = 37 kBq). Antibody to purified toxin was prepared in rabbits using toxin cross-linked to itself. These antibodies, partially purified by 50% ammonium sulfate precipitation and Sepharose CL-6B column chromatography, produced one precipitation band with killer toxin and bound 125I-labeled toxin in a radioimmunoassay. The antibody preparation also bound with the toxins from another K1 killer, A364A, and three chromosomal superkiller mutants derived from it.

L7 ANSWER 160 OF 184 MEDLINE ® on STN DUPLICATE 120
AN 1982041871 MEDLINE Full-text
DN PubMed ID: 7027641

TI Effect of growth factor deficiency on killer toxin sensitivity of Saccharomyces cerevisiae.
AU Jirku V; Cejkova A
SO Zeitschrift fur allgemeine Mikrobiologie, (1981) Vol. 21, No. 6, pp. 423-6.
Journal code: 0413631. ISSN: 0044-2208. L-ISSN: 0044-2208.
CY GERMANY, EAST: German Democratic Republic
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198112
ED Entered STN: 16 Mar 1990
Last Updated on STN: 16 Mar 1990
Entered Medline: 21 Dec 1981

L7 ANSWER 161 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1981:238706 BIOSIS Full-text
DN PREV198172023690; BA72:23690
TI ETHANOL PRODUCTION BY ZYMOMONAS-MOBILIS AND SACCHAROMYCES-UVARUM ON AFLA TOXIN CONTAMINATED AND AMMONIA DE TOXIFIED CORN.
AU NOFSINGER G W [Reprint author]; BOTHAST R J
CS NORTHERN REG RES CENT, AGRIC RES SCI AND EDUCATION ADMINISTRATION, US DEP OF AGRIC, PEORIA, IL, USA 61604
SO Canadian Journal of Microbiology, (1981) Vol. 27, No. 2, pp. 162-167.
CODEN: CJMIAZ. ISSN: 0008-4166.
DT Article
FS BA
LA ENGLISH
AB Z. mobilis demonstrated greater fermentative activity than S. uvarum during the 1st day in the fermentation of 2 lots of aflatoxin-contaminated corn and 2 corresponding lots of ammonia-detoxified corn. Final ethanol yields and conversion efficiencies were generally highest in Zymomonas fermentations of ammonia-detoxified corn. Aflatoxin levels in postfermentation solids from ammonia-detoxified corn all ranged below the USA Food and Drug Administration feedstuff guideline of < 20 ppb; the amount of aflatoxin in postfermentation solids from aflatoxin-contaminated corn was greater than in the initial corn.

L7 ANSWER 162 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1981:178146 BIOSIS Full-text
DN PREV198171048138; BA71:48138
TI PRIMARY EFFECTS OF YEAST SACCHAROMYCES-CEREVISIAE KILLER TOXIN.
AU DE LA PENA P [Reprint author]; BARROS F; GASCON S; RAMOS S; LAZO P S
CS DEP INTERFAC BIOQUIM, UNIV OVIEDO, SPAIN
SO Biochemical and Biophysical Research Communications, (1980) Vol. 96, No. 2, pp. 544-550.
CODEN: BBRCA9. ISSN: 0006-291X.
DT Article
FS BA
LA ENGLISH
AB Killer toxin from S. cerevisiae binds to sensitive cells immediately after addition to the cells. However, 50% mortality was obtained only after 40 min. Although it is thought that a lag phase is required for the killer to exert its action, experiments show that the killer starts affecting the cell immediately after binding. Thus, shortly after addition, the toxin was able to inhibit the transport of L-[3H]leucine as well as that of protons which are cotransported with this amino acid or with histidine. Moreover, killer toxin inhibited the pumping of protons to the medium by cells which were actively metabolizing glucose. These effects were a function of the concentration of toxin used. The results suggest that killer toxin acts by affecting the electrochemical proton gradient across the plasma membrane of yeast.

L7 ANSWER 163 OF 184 MEDLINE ® on STN DUPLICATE 121
AN 1981084883 MEDLINE Full-text
DN PubMed ID: 7004340
TI Effects of potassium and sodium ions on the killing action of a Pichia kluyveri toxin in cells of Saccharomyces cerevisiae.
AU Middelbeek E J; Crutzen Q H; Vogels G D
SO Antimicrobial agents and chemotherapy, (1980 Oct) Vol. 18, No. 4, pp. 519-24.
Journal code: 0315061. ISSN: 0066-4804. L-ISSN: 0066-4804.
Report No.: NLM-PMC284041.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 198102
ED Entered STN: 16 Mar 1990
Last Updated on STN: 29 Jan 1999
Entered Medline: 24 Feb 1981
OSC.G 2 There are 2 MEDLINE records that cite this record
REM.CNT 13 There are 13 cited references available in MEDLINE for this document.
AB Loss of viability of toxin-treated cells of Saccharomyces cerevisiae SCF 1717 could be prevented in the period before they altered physiologically if cells were incubated in media with a suitable concentration of potassium (0.08 to 0.13 M) and hydrogen ions (pH 6.2 to 6.7). Incorporation of higher amounts of potassium chloride in the media had a pronounced negative effect on cell survival, particularly when the pH of the medium was lowered. Replacement of KCl by NaCl in the plate media was even more deleterious to toxin-treated cells and, in contrast with potassium, low concentrations of sodium ions could not sustain recovery of cells. Complete recovery of a toxin-treated cell suspension required an incubation of 3 h in a suitable medium. The recovery process was blocked by cycloheximide.

L7 ANSWER 164 OF 184 MEDLINE ® on STN DUPLICATE 122
AN 1982055942 MEDLINE Full-text
DN PubMed ID: 7029035
TI Colonization and pathogenicity of Saccharomyces cerevisiae, MC16, in

mice and cynomolgus monkeys after oral and intravenous administration.
AU Maejima K; Shimoda K; Morita C; Fujiwara T; Kitamura T
SO Japanese journal of medical science & biology, (1980 Oct) Vol. 33, No. 5, pp. 271-6.
Journal code: 0243706. ISSN: 0021-5112. L-ISSN: 0021-5112.
CY Japan
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
EM 198201
ED Entered STN: 16 Mar 1990
Last Updated on STN: 16 Mar 1990
Entered Medline: 7 Jan 1982
OSC.G 1 There are 1 MEDLINE records that cite this record
AB Saccharomyces cerevisiae, an ascomycetous yeast and a candidate for a host-vector system in recombinant DNA experiments, was examined for its pathogenicity and colonization in mice and cynomolgus monkeys as the models of the biological containment level. Adult mice given perorally with 5.5 or 2.4X10(7) cells of MC16 strain S. cerevisiae excluded them rapidly and no colonization of the cells in the abdominal organs, lymph nodes or gastrointestinal wall was demonstrated. No change in the fecal flora was observed. Cynomolgus monkeys after peroral administration of 4.9X10(7) or 7.8X10(8) cells showed a similar tendency of rapid exclusion and the lack of colonization. Cortisone acetate treatment had no significant effect. Intravenous administration of 3.9X10(7) yeast cells had no pathogenic effect and no viable yeast cell was detected in the blood. The biological containment level of S. cerevisiae was suggested to be not lower than that of E. coli K12 B1 level, and the possibility of achieving the B2 level was also suggested.

L7 ANSWER 165 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1981:58624 BIOSIS Full-text
DN PREV198120058624; BR20:58624
TI MUTAGENICITY AND TOXICITY OF CADMIUM ON SACCHAROMYCES-CEREVISIAE.
AU EGER M [Reprint author]; DOKO J
CS INST MED RES OCCUP HEALTH, ZAGREB
SO Mutation Research, (1980) Vol. 74, No. 3, pp. 208.
Meeting Info.: 9TH ANNUAL MEETING OF THE EUROPEAN ENVIRONMENTAL MUTAGEN SOCIETY, MARKASKA, YUGOSLOVIA, SEPT. 30-OCT. 5, 1979. MUTAT RES.
CODEN: MUREAV. ISSN: 0027-5107.
DT Conference; (Meeting)
FS BR
LA ENGLISH

L7 ANSWER 166 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 1981016650 EMBASE Full-text
TI Mutagenicity and toxicity of cadmium on Saccharomyces cerevisiae.
AU Eger, M.; Doko, J.
CS Inst. Med. Res. Occupational Hlth, Zagreb, Yugoslavia.
SO Mutation Research, (1980) Vol. 74, No. 3, pp. 78.
CODEN: MUREAV
CY Netherlands
DT Journal
FS 022 Human Genetics
037 Drug Literature Index
LA English
ED Entered STN: 9 Dec 1991
Last Updated on STN: 9 Dec 1991

L7 ANSWER 167 OF 184 MEDLINE ® on STN DUPLICATE 123
AN 1979127581 MEDLINE Full-text
DN PubMed ID: 33806
TI Yeast killer toxin: purification and characterisation of the protein toxin from Saccharomyces cerevisiae.
AU Palfree R G; Bussey H
SO European journal of biochemistry / FEBS, (1979 Feb 1) Vol. 93, No. 3, pp. 487-93.
Journal code: 0107600. ISSN: 0014-2956. L-ISSN: 0014-2956.
CY GERMANY, WEST: Germany, Federal Republic of
DT (COMPARATIVE STUDY)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 197905
ED Entered STN: 15 Mar 1990
Last Updated on STN: 6 Feb 1995
Entered Medline: 16 May 1979
OSC.G 26 There are 26 MEDLINE records that cite this record
AB Killer toxin from killer strains of Saccharomyces cerevisiae was isolated from concentrates of extracellular medium by precipitation in poly(ethylene glycol) and chromatography through glyceryl-controlled-pore glass. The toxin migrated as a single protein band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. A molecular weight of 11470 was determined for the toxin protein from its electrophoretic mobility and amino acid composition. Gel filtration of the active toxin indicated that the 11,470-Mr monomer was the active unit. Electrophoretic comparison of extracellular concentrates from a killer strain and an isogenic non-killer showed the presence of the toxin protein only in the killer-derived material. The activity of the toxin was most stable between pH 4.2 and 4.6. At 30 degrees C toxin from a superkiller strain was more stable than that from a normal killer.

L7 ANSWER 168 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1980:288387 BIOSIS Full-text
DN PREV198070080883; BA70:80883
TI MERCURIAL TOXICITY IN YEAST SACCHAROMYCES-CEREVISIAE GLUCOSE UPTAKE GLYCOLYTIC AND FERMENTATIVE FUNCTIONS REMAIN UNIMPAIRED.
AU BRUNKER R L [Reprint author]
CS DEP BIOL SCI, ENVIRON STUD INST, DREXEL UNIV, PHILADELPHIA, PA 19104, USA

SO Microbios, (1979) Vol. 26, No. 105-106, pp. 147-152.
CODEN: MCBIA7. ISSN: 0026-2633.
DT Article
FS BA
LA ENGLISH
AB Yeast cells (Saccharomyces cerevisiae) were placed in association with sufficient concentrations of culture mercurials (as HgCl2) to arrest their uptake of culture dissolved O2. They were subsequently employed as substrates to test the status of membrane glucose transport mechanisms, the glycolytic-metabolic pathway, and the fermentative capabilities of Hg-stressed cells. These cells retain their glucose uptake capabilities as evidenced by their depletion of glucose in suspension fluids to levels similar to those of control cell suspensions; these cells could produce amounts of CO2 and ethanol which were similar to control cell productions. Mercurial toxicity evidently is not the result of an overall disruptive effect of cellular reactions or membrane functions but results from a specific lesion or lesions within the catabolic metabolism pathway inhibiting reactions that normally occur after glycolysis.

L7 ANSWER 169 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1978:78184 BIOSIS Full-text
DN PREV197815021684; BR15:21684
TI PURIFICATION AND PROPERTIES OF THE YEAST KILLER TOXIN FROM SACCHAROMYCES-CEREVISIAE.
AU PALFREE R G E; BUSSEY H
SO Abstracts of the Annual Meeting of the American Society for Microbiology, (1978) Vol. 78.1978, pp. 104.
CODEN: ASMACK. ISSN: 0094-8519.
DT Article
FS BR
LA Unavailable

L7 ANSWER 170 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1979:26514 BIOSIS Full-text
DN PREV197916026514; BR16:26514
TI CYTOCHROME P-450 AND THE ACTIVATION OF AFLA TOXIN B-1 IN SACCHAROMYCES-CEREVISIAE.
AU CALLEN D F; PHILPOT R M; ONG T-M
SO Mutation Research, (1978) Vol. 53, No. 1, pp. 85.
CODEN: MUREAV. ISSN: 0027-5107.
DT Article
FS BR
LA Unavailable

L7 ANSWER 171 OF 184 MEDLINE ® on STN DUPLICATE 124
AN 1977118451 MEDLINE Full-text
DN PubMed ID: 320190
TI Mode of action of yeast toxins: energy requirement for Saccharomyces cerevisiae killer toxin.
AU Skipper N; Bussey H
SO Journal of bacteriology, (1977 Feb) Vol. 129, No. 2, pp. 668-77.
Journal code: 2985120R. ISSN: 0021-9193. L-ISSN: 0021-9193.
Report No.: NLM-PMC234996.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS MEDLINE; Priority Journals
EM 197704
ED Entered STN: 13 Mar 1990
Last Updated on STN: 6 Feb 1998
Entered Medline: 15 Apr 1977
OSC.G 9 There are 9 MEDLINE records that cite this record
REM.CNT 27 There are 27 cited references available in MEDLINE for this document.
AB The role of the energy status of the yeast cell in the sensitivity of cultures to two yeast toxins was examined by using 12K release from cells as a measure of toxin action. The Saccharomyces cerevisiae killer toxin bound to sensitive cells in the presence of drugs that interfered with the generation or use of energy, but it was unable to efflux 12K from the cells under these conditions. In direct contrast, the Torulopsis glabrata pool efflux-stimulating toxin induced efflux of the yeast 42K pool was insensitive to the presence of energy poisons in cultures. The results indicate that an energized state, maintained at the expense of adenosine 5'-triphosphate from either glycolytic or mitochondrial reactions, is required for the action of the killer toxin on the yeast cell.

L7 ANSWER 172 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1978:56479 BIOSIS Full-text
DN PREV197814056479; BR14:56479
TI EFFECT OF GLUCOSE ON COPPER UPTAKE AND TOXICITY IN SACCHAROMYCES-CEREVISIAE.
AU ROSS I S
SO Bulletin of the British Mycological Society, (1977) Vol. 11, No. 2, pp. 142.
CODEN: BMYBAG. ISSN: 0007-1528.
DT Article
FS BR
LA Unavailable

L7 ANSWER 173 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1978:117443 BIOSIS Full-text
DN PREV197865004443; BA65:4443
TI EFFECT OF GLUCOSE ON COPPER UPTAKE AND TOXICITY IN SACCHAROMYCES-CEREVISIAE.
AU ROSS I S [Reprint author]
CS DEP BIOL, UNIV KEELE, KEELE, STAFFS, ENGL, UK
SO Transactions of the British Mycological Society, (1977) Vol. 69, No. 1, pp. 77-82.

CODEN: BMSTA6. ISSN: 0007-1536.
DT Article
FS BA
LA ENGLISH
AB Cu uptake by and toxicity to S. cerevisiae Hansen is greatly enhanced by glucose. Uptake and toxicity are markedly reduced at low temperature (1-2° C) and the enhancement by glucose abolished. Energy dependent transport of Cu is indicated but further investigation was precluded by high toxicity of very low Cu concentrations.

L7 ANSWER 174 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1976:192299 BIOSIS Full-text
DN PREV197662022299; BA62:22299
TI THE AMINO-ACID SEQUENCE OF PUROTHIONIN A A LETHAL TOXIC PROTEIN FOR BREWERS YEASTS FROM WHEAT.
AU OHTANI S; OKADA T; KAGAMIYAMA H; YOSHIZUMI H
SO Agricultural and Biological Chemistry, (1975) Vol. 39, No. 11, pp. 2269-2270.
CODEN: ABCHA6. ISSN: 0002-1369.
DT Article
FS BA
LA Unavailable

L7 ANSWER 175 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1974:122624 BIOSIS Full-text
DN PREV197457022324; BA57:22324
TI EFFECTS OF ETHIDIUM BROMIDE ON THE PRODUCTION OF FACTORS WITH TOXO HORMONAL ACTIVITY FOR SACCHAROMYCES-CEREVISIAE.
AU COSTA V; BLESA M C; OLIVARES J; MONTOYA E
SO Cuadernos de Ciencias Biologicas Universidad de Granada, (1972) Vol. 2, No. 1, pp. 59-61.
DT Article
FS BA
LA Unavailable

L7 ANSWER 176 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1970:217754 BIOSIS Full-text
DN PREV197051127754; BA51:127754
TI FUNGICIDAL ACTIVITY OF N 3 CHLOROPHENYL AND N-3 NITROPHENYL ITACON IMIDES AGAINST SACCHAROMYCES-CARLSBERGENSIS AND PATHOGENIC FUNGI.
AU GROVER R K
SO Acta Phytopathologica Academiae Scientiarum Hungaricae, (1969) Vol. 4, No. 2-3, pp. 203-212.
CODEN: APYPBZ. ISSN: 0001-6780.
DT Article
FS BA
LA Unavailable

L7 ANSWER 177 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1967:103165 BIOSIS Full-text
DN PREV19674800103167; BA48:103167
TI Modified ribosomes conferring resistance to cycloheximide in mutants of Saccharomyces cerevisiae. [Actidione toxicity].
AU COOPER, D.; BANTHORPE, D. V.; WILKIE, D.
CS Dep Bot., Univ. Coll., London, Engl., UK
SO J MOL BIOL, (1967) Vol. 26, No. 2, pp. 347-350.
DT Article
FS BA
LA Unavailable
ED Entered STN: May 2007
Last Updated on STN: May 2007

L7 ANSWER 178 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1965:6524 BIOSIS Full-text
DN PREV19654600006525; BA46:6525
TI Toxicity studies with yeasts [Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans, Helix pomat].
AU KOBAYASHI, GEORGE SHOICHIRO
SO DISS ABSTR, (1964) Vol. 25, No. 1, pp. 30.
DT Article
FS BA
LA Unavailable
ED Entered STN: May 2007
Last Updated on STN: May 2007
AB At least 2 factors play a role in the induction of immediate febrile response. The very physical nature of the yeast cell contributes to a response in a manner similar to that seen when the nonbiological polystyrene latex particles are injected. Secondly there is a truly pyrogenic substance, extractable by the hot phenol method, which is capable of inducing fever.

L7 ANSWER 179 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1964:101297 BIOSIS Full-text
DN PREV19644500101304; BA45:101304
TI The pathogenicity of Saccharomyces and personal case reports [French summ.J.
Original Title: I pathogonos drasis ton saccharomykiton kai perigrafi idion periptoseon [French summ.J.
AU STYLIANEA, A.
SO ACTA MICROBIOL HELL, (1963) Vol. 8, No. 3/4, pp. 161-107.
DT Article

FS BA
LA Unavailable
ED Entered STN: May 2007
Last Updated on STN: May 2007
AB From a case of onychia and a case of gastroenteritis Saccharomyces cerevisiae was isolated.

L7 ANSWER 180 OF 184 EMBASE COPYRIGHT (c) 2013 Elsevier B.V. All rights reserved on STN
AN 0047758825 EMBASE Full-text
TI Studies on sensitivity to toxic agents of saccharomyces sake (japanese).
AU Takai, S.
SO BIOL. J. NARA WOM. UNIV., (1962) Vol. 12, pp. 478-484.
DT Journal; Article
FS CLASSIC
LA English
SL English
ED Entered STN: Jun 2010
Last Updated on STN: Jun 2010
AB A respiratory deficient clone of Saccharomyces sake was induced by treatment with basic dyes, acriflavin, /xirarosaniline, pyronine. amethyst violet and cationic detergents. It was established that there are intimate interrelationships between sensitivity to each drug and the appearance of respiratory-deficient mutants, and between the chemical structures of the drugs and the permeabilities of the drugs into the yeast cells.

L7 ANSWER 181 OF 184 MEDLINE ® on STN DUPLICATE 125
AN 1960188653 MEDLINE Full-text
DN PubMed ID: 14435923
TI Metabolic effects of isonicotinic acid hydrazid (INH) toxicity in Saccharomyces cerevisiae.
AU RAJAGOPALAN K V; UDAYACHANDER M; SARMA P S
SO Archives of biochemistry and biophysics, (1960 Apr) Vol. 87, pp. 184-7.
Journal code: 0372430. ISSN: 0003-9861. L-ISSN: 0003-9861.
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS OLDMEDLINE; NONMEDLINE
EM 199811
ED Entered STN: 16 Jul 1999
Last Updated on STN: 16 Jul 1999
Entered Medline: 1 Nov 1998

L7 ANSWER 182 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1960:60795 BIOSIS Full-text
DN PREV196035000060818; BA35:60818
TI Metabolic effects of isonicotinic acid hydrazide (INH) toxicity in Saccharomyces cerevisiae.
AU RAJAGOPALAN, K. V.; UDAYACHANDER, M.; SARMA, P. S.
CS Univ Biochem. Lab., Madras, India
SO ARCH BIOCHEM AND BIOPHYS, (1960) Vol. 87, No. 2, pp. 184-187.
DT Article
FS BA
LA Unavailable
ED Entered STN: May 2007
Last Updated on STN: May 2007
AB Cells of S. cerevisiae grown in presence of growth-inhibitory levels of INH had higher sulfanilamide acetylating activity and enhanced coenzyme A levels. These data are taken to indicate an adaptive response on the part of the organism to overcome the toxicity of the drug by acetylating it. A concomitant increase in the total cholesterol content-of the cells was observed also. The results are discussed in relation to the incidence of peripheral neuritis induced by INH toxicity in experimental animals and clinical cases. ABSTRACT AUTHORS: Authors

L7 ANSWER 183 OF 184 MEDLINE ® on STN DUPLICATE 126
AN 1959035245 MEDLINE Full-text
DN PubMed ID: 13605791
TI Saccharomyces carlsbergenesis, possibly a pathogenic.
AU REIERSOL S; HOEL J
SO Acta pathologica et microbiologica Scandinavica, (1958) Vol. 44, No. 3, pp. 313-8.
Journal code: 7508471. ISSN: 0365-5555. L-ISSN: 0365-5555.
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS OLDMEDLINE; NONMEDLINE
OS CLML5935-35365-108-231-631
EM 200007
ED Entered STN: 25 Aug 2000
Last Updated on STN: 25 Aug 2000
Entered Medline: 1 Jul 2000
OSC.G 1 There are 1 MEDLINE records that cite this record

L7 ANSWER 184 OF 184 BIOSIS COPYRIGHT (c) 2013 The Thomson Corporation on STN
AN 1933:3819 BIOSIS Full-text
DN PREV19330700003835; BA07:3835
TI Un cas de blastomycose inveteree trans-missible au cobaye, due a un Saccharomyces pathogene (Saccharomyces jadini n. sp.).
AU SARTORY, A.; SARTORY, R.; WEILL, J.; MEYER, J.
SO COMPT REND ACAD SCI [PARIS], (1932) Vol. 194, No. 19, pp. 1688-1690.
DT Article
FS BA
LA Unavailable
ED Entered STN: May 2007
Last Updated on STN: May 2007
AB A yeast, Sac-charomyces jadini, was isolated from an abscess in the sacral region, the cells of which were round, 4-5u, in diameter, and which showed numerous asci with 2-4 ascospores; it did not liquefy gelatin or attack

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albumin, it coagulated milk without peptonization, and fermented glucose and maltose but not lactose or levulose. Intracardiac inoculation of guinea pigs caused the appearance of lymphatic nodules, hypertrophy of the spleen and liver, and abscesses in these organs and in the lungs. The urine reduced Fehling's solution. Death occurred in 5-7 mos. Retro-cultures were positive. The patient was treated by intradermic injections of CuSO4, neoidipine, insulin and ultraviolet radiation. ABSTRACT AUTHORS: I. M. Korr

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Food

Agency Response Letter GRAS Notice No. GRN 000120

CFSAN/Office of Food Additive Safety
June 30, 2003

Robert Biwersi
Lesaffre Yeast Corporation
433 East Michigan Street
Milwaukee, WI 53202

Re: GRAS Notice No. GRN 000120

Dear Mr. Biwersi:

The Food and Drug Administration (FDA) is responding to the notice, dated January 2, 2003, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on January 7, 2003, filed it on January 10, 2003, and designated it as GRAS Notice No. GRN 000120.

The subject of the notice is *Saccharomyces cerevisiae* strain ML01 (*S. cerevisiae* strain ML01) carrying a gene encoding malolactic enzyme from *Oenococcus oeni* and a gene encoding malate permease from *Schizosaccharomyces pombe*. The notice informs FDA of the view of Lesaffre Yeast Corporation (Lesaffre) that *S. cerevisiae* strain ML01 is GRAS, through scientific procedures, for use in winemaking as a yeast starter culture for grape must fermentation. Lesaffre recommends using between 0.1 to 0.2 grams of active dry yeast per liter of wine.

Lesaffre describes generally available information about traditional manufacturing processes for the production of wine from grapes. These processes include the harvesting, de-stemming and crushing of grapes (resulting in must), the separation of the juice from the skins and seeds, one or more distinct types of microbial fermentation, clarification, stabilization, and bottling. Winemakers may vary the sequence of operational steps or modify procedures, depending upon the desired characteristics and nature of the wine.

Lesaffre describes generally available information about two distinct fermentation processes (i.e., alcoholic fermentation and malolactic fermentation) that occur either through the action of microorganisms that already are present on the grapes or through the action of microorganisms that are specifically added by the winemaker. Alcoholic fermentation (i.e., a process whereby the sugars glucose and fructose are converted to ethanol) is mediated by metabolic pathways associated with yeast (usually *S. cerevisiae* or closely related species). Malolactic fermentation (i.e., a process whereby the dicarboxylic acid malic acid is decarboxylated to the monocarboxylic acid lactic acid) is mediated by lactic acid bacteria through the combined action of a protein (called malate permease) that transports malic acid from the wine into the bacteria and an enzyme (called malolactic enzyme) that converts the malic acid to lactic acid. Because malolactic fermentation reduces the number of carboxylic acid groups on organic acids present in the wine, it reduces the acidity of the must. Although alcoholic fermentation is an inherent process associated with all winemaking, malolactic fermentation is a secondary process that may or may not be induced by the winemaker, depending on the desired characteristics and nature of the wine.

Lesaffre describes generally available information about clarification of wine, which can occur either at the end of the alcoholic fermentation or after the wine has been kept on the lees (the sediment formed by spent yeast cells and grape particulate matter). Wine clarification encompasses the removal of solid particles in the wine via gravity or centrifugation and subsequent elimination of the sediment or pellet. When clarification occurs at the end of fermentation, the clarification process removes most yeast cells. When the wine is kept on the lees before clarification, the yeast cells undergo autolysis, which releases cellular material that ultimately is degraded through the action of enzymes such as proteases.

Lesaffre describes generally available information about stabilization processes, which differ depending on whether the wine is a white wine or a red wine. For white wine, stabilization involves removing proteins via filtration with bentonite. For red wine, stabilization involves adding gelatin or egg white albumin to precipitate colloidal structures that include tannin-protein complexes. Prior to bottling, most wines undergo filtration (e.g., with diatomaceous earth, cellulose filters, or membrane filters) that eliminates any remaining yeast cells.

Lesaffre describes published articles about bioengineered strains of *S. cerevisiae*, including strains of *S. cerevisiae* that have been modified to conduct malolactic fermentation. Lesaffre notes that the use of bioengineered strains

that can conduct both alcoholic and malolactic fermentation eliminates the need for separate additions of two distinct microorganisms (i.e., yeast and lactic acid bacteria).

Lesaffre describes the development of its own bioengineered strain of *S. cerevisiae*. The host strain, *S. cerevisiae* strain S92, was isolated from the Champagne region in France and is closely related or identical to commercial strains commonly used in winemaking. The microbial source of malate permease (i.e., *Schizosaccharomyces pombe*), is a yeast[1] that was first isolated from African beer and has frequently been found in sugar-containing products in tropical and sub-tropical regions and in grape must and cider in moderate climates. The microbial source of malolactic enzyme (i.e., *Oenococcus oeni*) is a lactic acid bacterium that has been isolated from wines and related habitats such as wineries and vineyards. It is the preferred, and most commonly used, lactic acid bacterium for malolactic fermentation of wines. The malate permease is a 49 kDa protein with a hydrophobicity profile typical of membrane transport proteins. It contains a peptide sequence (composed of proline, glutamic acid, serine and threonine) that characterizes proteins with a rapid turnover. The malolactic enzyme is a dimer with a total molecular weight of approximately 130 kDa.

Lesaffre describes the construction of an integration cassette that contains genes encoding malate permease from *S. pombe* and the malolactic enzyme from *O. oeni*, regulatory sequences associated with the expression of these genes, and sequences used for integration into an appropriate chromosomal site in *S. cerevisiae* strain S92. Lesaffre also describes the transformation strategy that it used to reduce the numbers of potentially transformed yeasts that needed to be screened for the successful integration of the integration cassette. This strategy involved co-transformation of *S. cerevisiae* strain S92 with a plasmid (pUT322) that carries a selectable marker conferring resistance to the antibiotic phleomycin and was based on the hypothesis that cells transformed with plasmid pUT322 are more likely to also have been transformed with the integration cassette. Using this strategy, Lesaffre first screened transformed yeast for resistance to phleomycin and then screened the selected phleomycin-resistant yeast for the ability to produce lactic acid. Lesaffre obtained a phleomycin-sensitive isolate and confirmed that it is free of plasmid pUT332 sequences. Lesaffre designated this strain as ML01.

Based on DNA analysis, Lesaffre concluded that the chromosomal patterns of *S. cerevisiae* strains S92 and ML01 are the same except for the presence of the integration cassette. Lesaffre found that the integration cassette remained stably incorporated after 100 generations. Lesaffre also found that *S. cerevisiae* strain ML01 functions as intended in that it efficiently degrades malic acid. Based on studies that evaluated yeast physiology under different culture conditions, Lesaffre concluded that *S. cerevisiae* strain ML01 has the same growth kinetics, fermentation rate, and ethanol yield as *S. cerevisiae* strain S92 under winemaking conditions and that uptake and utilization of malic acid did not confer a growth advantage to *S. cerevisiae* strain ML01.

Lesaffre describes the method for routine production of *S. cerevisiae* strain ML01 and notes that this method is based on well-established procedures for the production of active dry yeast. The yeast is grown primarily under aerobic conditions to promote yeast propagation rather than alcohol production. The yeast is harvested via centrifugation and is subsequently dewatered with a rotary vacuum filter, processed through an extruder, and dried, resulting in active dry yeast. The yeast is packaged in vacuum foil pouches prior to shipping.

Lesaffre discusses potential dietary intake of *S. cerevisiae* strain ML01 and of the proteins that Lesaffre has introduced into that strain. Lesaffre considers that exposure to the yeast itself or to the newly introduced proteins would be negligible because the processing procedures used in winemaking remove intact yeast cells, debris associated with autolyzed yeast cells, and proteins released during autolysis of yeast cells.

Based on the information provided by Lesaffre, as well as other information available to FDA, the agency has no questions at this time regarding Lesaffre's conclusion that *Saccharomyces cerevisiae* strain ML01 is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *S. cerevisiae* strain ML01. As always, it is the continuing responsibility of Lesaffre to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

[1]Although malolactic fermentation is usually mediated by lactic acid bacteria, Lesaffre chose a yeast (rather than a lactic acid bacterium) as a source of the permease, because the permease must function in the membrane of the yeast *S. cerevisiae*.

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Food

Agency Response Letter GRAS Notice No. GRN 000175

CFSAN/Office of Food Additive Safety

January 6, 2006

Hendrik J.J. van Vuuren, Ph.D.
First Venture Technologies Corporation.
Suite 1500
885 West Georgia Street
Vancouver, British Columbia, V6C 3E8
Canada

Re: GRAS Notice No. GRN 000175

Dear Dr. van Vuuren:

The Food and Drug Administration (FDA) is responding to the notice, dated July 5, 2005, that First Venture Technologies Corporation (First Venture) submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received this notice on July 7, 2005, filed it on July 12, 2005, and designated it as GRN No. 000175.

The subject of the notice is *Saccharomyces cerevisiae* strain ECMo01, which carries a recombinant genetic insert. The genetic insert is composed of three elements, *DUR1,2* gene, a promoter and a terminator, all derived from different strains of *S. cerevisiae*. The promoter and the terminator are necessary for the proper expression of the *DUR1,2* gene. The *DUR1,2* gene encodes *S. cerevisiae* urea amidolyase enzyme (urea amidolyase). Strain ECMo01 was developed by inserting the recombinant genetic insert into the *S. cerevisiae* parental strain Davis 522, which is commonly used in the wine industry.

First Venture states that strain Davis 522 contains its own *DUR1,2* gene, which is not normally active during alcoholic fermentation. Thus, the purpose of creating *S. cerevisiae* strain ECMo01 is to increase the expression of *S. cerevisiae* urea amidolyase. The urea amidolyase catalyzes the hydrolysis of urea that is produced by *S. cerevisiae* during alcoholic fermentation. Urea is a precursor of ethyl carbamate, a suspected carcinogen in humans, which is formed in the wine from the reaction of urea and ethanol. Hydrolysis of urea by urea amidolyase significantly reduces the potential for formation and accumulation of ethyl carbamate in the wine.

The notice informs FDA of the view of First Venture that strain ECMo01 is GRAS, through scientific procedures, for use as a starter culture in alcoholic beverage fermentation. First Venture has determined that strain ECMo01 is substantially equivalent to strain Davis 522 from which it was derived, except for the fully characterized introduced genetic insert and expressed levels of *S. cerevisiae* urea amidolyase.

First Venture describes published and unpublished information about the development of its strain ECMo01. In the notice, First Venture describes the details of the construction of the genetic insert, as well as its molecular characterization and stability assessment in strain ECMo01. First Venture states that the genetic insert does not contain any undesired sequences and it is genetically stable. First Venture states that strain Davis 522 is commonly used in industry to obtain wine active dry yeast and it has never been shown to produce allergenic or toxic substances.

First Venture states that they carried out risk assessments related to the presence of potential unintended gene products as a result of the genetic modification using computer-based sequence analyses. Their sequence analyses revealed a theoretical possibility of production of three additional proteins due to the integration of the genetic insert in strain ECMo01. First Venture asserts that further sequence analyses revealed that the production of these additional proteins is unlikely, and even in the extremely unlikely event of production of these proteins, there should be no concern because their amino acid sequences will be similar to those found in strain Davis 522, which has never been shown to produce unwanted products.

First Venture describes generally available information on wine making procedures and states that use of the strain ECMo01 does not lead to any changes in these procedures or in the composition of the wine. First Venture states that urea amidolyase is present in all wine yeast cells currently used by the wine industry. Therefore, the only significant difference expected between wines produced using strain Davis 522 and wines produced using strain ECMo01, is the possible presence of minor amounts of recombinant urea amidolyase. However, the clarification and stabilization processes, which are obligatory in commercial winemaking, will remove most of this

enzyme and its larger fragments.

First Venture describes generally available information on the clarification and stabilization of wine and explains how the remaining urea amidolyase could be removed during these processes. During wine clarification, solid particles may be allowed to settle by gravity or centrifugation. First Venture states that this clarification process removes most of the yeast cells. When the wine is kept on the lees for ageing, the yeast cells undergo autolysis, releasing cellular materials that are degraded through the action of enzymes. Further, white wines can be treated with bentonite to remove all remaining protein fractions. In red wines, tannins associate with proteins. This protein-tannin complex can be precipitated by adding gelatin or egg white albumin. First Venture states that prior to bottling most wines undergo filtration (e.g., with diatomaceous earth, cellulose filters, or membrane filters) that also eliminates any remaining yeast cells. First Venture concludes that use of the strain ECMo01 will not result in any additional urea amidolyase in wines at the time they are consumed.

Based on the information provided by First Venture, as well as other information available to FDA, the agency has no questions at this time regarding First Venture's conclusion that *Saccharomyces cerevisiae* strain ECMo01 encoding *S. cerevisiae* urea amidolyase enzyme is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *Saccharomyces cerevisiae* strain ECMo01. As always, it is the continuing responsibility of First Venture to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Laura M. Tarantino, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Food

Agency Response Letter GRAS Notice No. GRN 000239

CFSAN/Office of Food Additive Safety
July 22, 2008

Donald J. Cox, Ph.D.
Vice President of R&D
and Business Development
Healthcare Group
Biothera, Inc
3388 Mike Collins Drive
Eagan, MN 55121

Re: GRAS Notice No. GRN 000239

Dear Dr. Cox:

The Food and Drug Administration (FDA) is responding to the notice, dated January 11, 2008, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on January 16, 2008, filed it on January 24, 2008, and designated it as GRAS Notice No. GRN 000239.

The subject of the notice is bakers yeast beta-glucan. The notice informs FDA of the view of Biothera, Inc. (Biothera) that bakers yeast beta-glucan is GRAS, through scientific procedures, for use as an ingredient in a variety of food products including baked goods and baking mixes, beverages and beverage bases, cereals and cereal products, dairy product analogs, milk and milk products, plant protein products, processed fruits and fruit juices, soft candy, soups and soup mixes at a level of up to 200 milligrams per serving.

21 CFR 101.4 states that all ingredients must be declared by their common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. FDA's Office of Nutrition, Labeling, and Dietary Supplements (ONLDS) in the Center for Food Safety and Applied Nutrition, considers the name "bakers yeast beta-glucan" to be an appropriate name for this substance. ONLDS considered that this name adequately and accurately describes the substance and is specific to beta-glucan derived from bakers yeast, understanding that beta-glucans can be derived from other sources, including cereal grains and that the composition of such beta-glucans may vary depending on the source material. ONLDS also notes that "bakers yeast beta-glucan" is consistent with other terms used in our GRAS and food additive regulations for substances derived from bakers yeast, such as "bakers yeast glycan," "bakers yeast protein," and "bakers yeast extract." ONLDS notes that Biothera makes a statement about yeast sensitivity/allergy and considers that "bakers yeast beta-glucan" provides clear and accurate information to such consumers.

As part of its notice, Biothera includes the report of a panel of individuals (Biothera's GRAS panel) who evaluated the data and information that are the basis for Biothera's GRAS determination. Biothera considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Biothera's GRAS panel evaluated identity, method of manufacture, product specifications, estimated daily intake, and generally available safety data for the intended use of bakers yeast beta-glucan as a food ingredient. Based on this review, Biothera's GRAS panel concluded that bakers yeast beta-glucan that meets its established food grade specifications and produced in accordance with current good manufacturing practices (cGMP), is GRAS under the conditions of its intended use.

Biothera describes the identity of bakers yeast beta-glucan. Bakers yeast beta-glucan is a beige-to-tan colored powder obtained from bakers yeast (*Saccharomyces cerevisiae*) cell walls. The product contains at least 70% insoluble beta-(1,3),(1,6)-D-glucan. In addition, small amounts of beta-1,6-glucan, chitin, protein, and lipids are present in the product. Biothera produces bakers yeast beta-glucan in two forms: one with at least 70% beta-glucan, the other with at least 75% beta-glucan. Biothera uses only one manufacturing process to produce both forms of bakers yeast beta-glucan.

Biothera describes the method of manufacture and provides specifications for bakers yeast beta-glucan. Biothera manufactures bakers yeast beta-glucan from food-grade bakers yeast (*S. cerevisiae*) grown at Biothera's production plant under controlled conditions in stainless steel fermentation vessels. To extract the cell walls, Biothera lyses the yeast cells using a thermal process and separates the cell wall component from the yeast extract using centrifugation. To strip the mannosylated cell wall proteins that are linked to the cell wall, the cell

wall isolate undergoes a caustic treatment. This step also removes residual cellular lipids trapped within the cell wall. Subsequently, the ingredient undergoes an acid treatment, which results in the depolymerization and deacetylation of chitin to form free glucosamine hydrochloride, resulting in the removal of most of the chitin. Next, the yeast wall slurry undergoes flash sterilization, followed by pH adjustment steps, resulting in a sterile solution with an approximate pH range of 5-6. The resulting mixture contains between 6 to 12% of the original solids. The mixture is then spray-dried. Once dry, the powder is sieved through a mesh sifter. Biothera provides product specifications for bakers yeast beta-glucan, including specifications for total carbohydrates (≥80%) composed primarily of beta-(1,3),(1,6)-glucan (≥70%), protein, fat, ash, moisture, lead, and microbiological specifications.

Biothera notes that small amounts of volatile organic compounds (methanol, ethanol, isopropanol, acetone and hexane) can be detected in the final bakers yeast beta-glucan product even though no organic solvents are used during the manufacturing process. Biothera determined that these compounds are the result of fatty acid decomposition during the manufacturing process. Based on their levels in the final product, Biothera concludes that the volatile organic compounds are not expected to produce adverse effects on human health.

Biothera estimates the all-user mean intake of bakers yeast beta-glucan from its intended use in the food categories listed above. These estimates were derived from the USDA Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996. Biothera estimates that the intake of bakers yeast beta-glucan under the conditions of its intended use would be ca. 413 milligrams per person per day (mg/p/d) (8.90 mg per kilogram body weight per day) (mg/kg bw/d) and the 90th percentile intake as ca. 827 mg/person/day (20.66 mg/kg bw/d).

Biothera asserts that the safety of bakers yeast beta-glucan is partly supported by the compositional similarity of the substance to bakers yeast glycan, which is listed in 21 CFR 172.898. Biothera notes that bakers yeast beta-glucan and bakers yeast glycan are both manufactured from cell walls of *S. cerevisiae*, are composed almost entirely of carbohydrates, and their specifications include similar limits for chemical and microbiological parameters.

Biothera summarizes published and unpublished rodent and human studies, including acute toxicity studies in rats and mice, a subchronic oral toxicity study in rats, and double-blind, placebo-controlled studies for 10 and 30 days in humans. Biothera notes that no adverse effects were observed in any of the studies. Biothera concludes that the rodents in the acute toxicity studies had no evidence of clinical chemistry or histopathological toxicity. In the subchronic oral toxicity study, the rats showed no evidence of systemic or gastrointestinal toxicity at the highest level (100 mg/kg bw/d) of bakers yeast beta-glucan. Biothera discusses the results of the human clinical studies, and concludes that levels up to 500 mg/p/d of bakers yeast beta-glucan were well-tolerated and that there were no significant differences in blood biochemistry parameters.

Standards of Identity

In the notice, Biothera states its intention to use yeast beta-glucan in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FFDCA)

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FFDCA to, among other things, add section 301(ll). Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Biothera's notice that bakers yeast beta-glucan is GRAS for use in a variety of food products, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing bakers yeast beta-glucan. Accordingly, this response should not be construed to be a statement that foods that contain bakers yeast beta-glucan, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Biothera, as well as other information available to FDA, the agency has no questions at this time regarding Biothera's conclusion that bakers yeast beta-glucan is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of bakers yeast beta-glucan. As always, it is the continuing responsibility of Biothera to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000239, as well as a copy of the information in this notice that conforms to the information in the proposed GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying on the homepage of OFAS (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

Laura M. Tarantino, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

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Food

Agency Response Letter GRAS Notice No. GRN 000260

CFSAN/Office of Food Additive Safety

March 9, 2009

Robert S. McQuate, Ph.D.
GRAS Associates, LLC
20482 Jacklight Lane
Bend, OR 97702-3074

Re: GRAS Notice No. GRN 000260

Dear Dr. McQuate:

The Food and Drug Administration (FDA) is responding to the notice, dated September 3, 2008, that you submitted on behalf of Cypress Systems, Inc. (CSI), in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on September 10, 2008, filed it on September 12, 2008, and designated it as GRAS Notice No. GRN 000260.

The subject of the notice is high-selenium yeast. The notice informs FDA of the view of CSI that high-selenium yeast is GRAS, through scientific procedures, for use as an ingredient in baked products, non-alcoholic beverages, breakfast cereals, grain products and pastas, milk products, processed fruit and fruit juices, processed vegetables and vegetable juices, commercial soups and soup mixes, and medical foods at levels yielding 5 micrograms (µg) selenium per serving.(1)

21 CFR 101.4 states that all ingredients must be declared by their common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. Our use of "high-selenium yeast" in this letter should not be considered an endorsement or recommendation of that term as an appropriate common or usual name for the purpose of declaring the substance in the ingredient statement of foods that contain that ingredient. Issues associated with labeling and the appropriate common or usual name of a food are the responsibility of the Office of Nutrition, Labeling, and Dietary Supplements (ONLDS) in the Center for Food Safety and Applied Nutrition.

As part of its notice, CSI includes the report of a panel of individuals (CSI's GRAS panel) who evaluated the data and information that are the basis for CSI's GRAS determination. CSI considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. CSI's GRAS panel evaluated information relevant to the identity and composition of high-selenium yeast, selenium toxicity, dietary exposure to selenium from high-selenium yeast, substantial equivalence of the yeast component to conventional yeast, and potential for allergenicity. Based on this review, CSI's GRAS panel concluded that high-selenium yeast, produced in accordance with good manufacturing practices and complying with appropriate food-grade specifications, is GRAS under the conditions of its intended use.

CSI discusses the identity and characteristics of high-selenium yeast. Food-grade high-selenium yeast produced from *Saccharomyces cerevisiae* (also known as baker's yeast) is a dry powder containing 1,140 – 1,260 µg of selenium per gram of yeast, present as a mixture of four chemically characterized organoselenium forms which account for approximately 85 percent of the selenium content.(2) The predominant form of selenium in the mixture is identified as selenomethionine (84 percent); the three other characterized forms of selenium are present at low levels and include γ-glutamyl-Se-methyl-Se-cysteine (0.5 percent), Se-adenosyl-Se-homocysteine (0.5 percent), and selenite (0.1 percent). The notifier states that comparison of chemical composition and yeast performance parameters establish that standard baker's yeast and high-selenium yeast are virtually the same except for the presence of increased levels of selenium in substitution for sulfur within the high-selenium yeast.

CSI describes the manufacturing methods for high-selenium yeast. High-selenium yeast is produced using *S. cerevisiae* as the yeast into which inorganic selenium, as sodium selenite, is incorporated. The continuous fermentation utilizes a medium with minimal sulfur and methionine levels to enhance selenium incorporation into the yeast. The chilled mineralized yeast cream is inactivated when pasteurized through a high temperature sterilization system to achieve food grade standards and subsequently spray dried to yield a uniformly homogeneous dry powder.

CSI provides specifications for food-grade high-selenium yeast with target levels for selenium (1,140 – 1,260 µg

selenium per gram of yeast).[3] Specifications also include physical/chemical target levels for protein, phosphorus, moisture, extraneous material, and inorganic selenium, as well as limitations on microbial contaminants and heavy metals.

CSI calculates an estimated daily intake (EDI) for selenium from the consumption of high-selenium yeast when added to foods in accordance with the intended conditions of use. CSI determines, on the basis of an estimated maximum number of daily servings of foods within each of the designated food categories, that the EDI would be less than 100 µg of selenium per person per day.

CSI discusses the safety of selenium and high-selenium yeast. The notifier provides summaries of published literature reviews and primary literature. The cited publications include consideration of both animal and human studies and consist of (1) evaluations of bioavailability, as well as absorption, distribution, metabolism and excretion of the relevant forms of selenium found in high-selenium yeast; (2) determinations of safe upper limits for selenium intake; and (3) evaluations of potential genotoxicity, reproductive toxicity, and teratogenicity associated with selenium (both inorganic and organic forms) and high-selenium yeast. The notifier further discusses the results of published epidemiological and clinical studies as corroborative evidence of the safe upper limits of selenium for human populations. CSI reports that the literature generally agrees that the safe upper limit of selenium intake is in the range of 300 to 400 µg selenium per day. On the basis of human experiences and associated testing, pertinent animal test results, anticipated human consumption levels, and germane supporting information, CSI concludes that high-selenium yeast, produced in accordance with good manufacturing practices and complying with appropriate food-grade specifications, is GRAS under the conditions of its intended use.

The notifier discusses a possible association between type-2 diabetes and selenium intake as well as possible allergenicity to the yeast used in the production of high-selenium yeast. The notifier concludes that there is a lack of evidence in the published scientific literature to support such associations and that consumption of high-selenium yeast does not constitute a safety concern for type-2 diabetes or for allergenicity.

Standards of Identity

In the notice, CSI states its intention to use high-selenium yeast in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Potential Labeling Issues

Under section 403(a) of the Federal Food, Drug, and Cosmetic Act (FFDCA), a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FFDCA lays out the statutory framework for the use of labeling claims that characterize the level of a nutrient in a food or that characterize the relationship of a nutrient to a disease or health-related condition. In describing the intended use of high-selenium yeast and in describing the information that CSI relies on to conclude that high-selenium yeast is GRAS under the conditions of its intended use, CSI raises a potential issue under these labeling provisions of the FFDCA. If products that contain high-selenium yeast bear any claims on the label or in labeling, such claims are the purview of the ONLDS in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety neither consulted with ONLDS on this labeling issue nor evaluated the information in your notice to determine whether it would support any claims made about high-selenium yeast on the label or in labeling.

Medical Foods

In its notice, CSI informs FDA that one intended use of high-selenium yeast is use in medical foods. Section 5(b) of the Orphan Drug Act (ODA) defines a medical food as a food that is formulated to be consumed or administered enterally under the supervision of a physician and that is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation. Section 403(q) of the FFDCA lays out the statutory framework for nutrition labeling of food products. Section 403(r) of the FFDCA lays out the statutory framework for health claims and nutrient content claims. Under section 403(q)(5)(A)(iv) of the FFDCA and FDA's implementing regulations in 21 CFR 101.9(j)(8), the requirements for nutrition labeling do not apply to medical foods as defined in section 5(b) of the ODA. Under section 403(r)(5)(A) of the FFDCA and FDA's implementing regulations in 21 CFR 101.13(q)(4)(ii) and 21 CFR 101.14(f)(2), the requirements for nutrient content claims and health claims, respectively, do not apply to medical foods as defined in section 5(b) of the ODA. For your information, FDA's response to CSI's notice that high-selenium yeast is GRAS for use in medical foods does not address the question of whether any particular food product that contains high-selenium yeast as an ingredient would be a medical food within the meaning of section 5(b) of the ODA and, thus, would be exempt from the requirements for nutrition labeling, nutrient content claims, and health claims.

Section 301(ll) of the FFDCA

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FFDCA to, among other things, add section 301(ll). Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of CSI's notice that high-selenium yeast is GRAS for use in the intended food categories, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing high-selenium yeast. Accordingly, this response should not be construed to be a statement that foods that contain high-selenium yeast, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by CSI, as well as other information available to FDA, the agency has no questions at this time regarding CSI's conclusion that high-selenium yeast is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of high-selenium yeast. As always, it is the continuing responsibility of CSI to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000260, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying via the FDA home page at http://www.fda.gov. To view or obtain an electronic copy of the text of the letter, follow the hyperlinks from the "Food" topic to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Sincerely,

Laura M. Tarantino, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

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[1]CSI states that foods intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, are excluded from the intended uses.

[2]CSI notes that 15 percent of the selenium content is uncharacterized due to analytical limitations. However, CSI concludes that numerous published human studies using high-selenium yeast, without apparent toxicity or noted adverse effects, overrides the compositional uncertainties. On this basis, CSI concludes that the compositional uncertainties do not impact safety considerations.

[3]CSI notes that commercial baker's yeast typically contains less than 5 µg of selenium per gram of yeast.

Links on this page:

Food

Agency Response Letter GRAS Notice No. GRN 000284

CFSAN/Office of Food Additive Safety

August 28, 2009

Gary L. Yingling
K & L Gates, LLP
1601 K Street NW
Washington, DC 20006-1600

Re: GRAS Notice No. GRN 000284

Dear Mr. Yingling:

The Food and Drug Administration (FDA) is responding to the notice, dated March 06, 2009, that you submitted on behalf of DSM Food Specialties (DSM) in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on March 10, 2009, filed it on March 12, 2009, and designated it as GRAS Notice No. GRN 000284.

The subject of the notice is bakers yeast mannoprotein. The notice informs FDA of the view of DSM that bakers yeast mannoprotein is GRAS, through scientific procedures, for use as a stabilizing agent in wines, at levels ranging from 50–400 milligrams per liter (mg/L), to prevent tartaric acid precipitation.

21 CFR 101.4 states that all ingredients must be declared by their common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. Our use of "bakers yeast mannoprotein" in this letter should not be considered an endorsement or recommendation of that term as an appropriate common or usual name for the purpose of declaring the substance in the ingredient statement of foods that contain that ingredient. Issues associated with labeling and the appropriate common or usual name of a food are the responsibility of the Office of Nutrition, Labeling, and Dietary Supplements in the Center for Food Safety and Applied Nutrition.

DSM describes the identity and composition of bakers yeast mannoprotein. Bakers yeast (*Saccharomyces cerevisiae*) mannoprotein contains a family of glycosylated proteins and peptides of varying structures and molecular weights (20–450 kilodaltons (kDa)) present in the yeast cell wall and periplasm. The polysaccharide chains of mannoprotein consist almost exclusively of α-1→6 linked mannose units with short α-1→2 and α-1→3 linked side chains. Some of these side chains may have phosphodiester linkages to other mannosyl residues. The carbohydrate content of bakers yeast mannoprotein is at least 60 percent; no less than 70 percent of the carbohydrate is mannose. DSM produces bakers yeast mannoprotein in two forms: a spray dried powder and a colloidal solution (containing approximately 20 percent dry matter).

DSM describes the method of manufacture of bakers yeast mannoprotein from food-grade yeast extract. The steps include micro-filtration of the yeast extract to remove high molecular weight proteinaceous material followed by ultrafiltration (UF) to concentrate the mannoprotein (greater than 20 kDa). The UF-concentrate is treated with the enzyme phosphodiesterase to hydrolyze residual yeast RNA, followed by a diafiltration step to remove low molecular weight moieties such as salts, amino acids, nucleotides, and sugars. The dilute mannoprotein fraction is then concentrated by UF, followed by ultra-high temperature (UHT) treatment (130–150 degrees Celsius) to inactivate residual enzyme. The UF concentrate is cooled and polish filtered to remove insoluble proteinaceous material formed during the UHT treatment. After filtration, the clear solution is either (1) spray dried and packed as a powder or (2) stabilized with 14 grams per liter of sodium bisulfite as a preservative and packed as a colloidal solution. Both finished products are stored at 4 degrees Celsius.

DSM provides chemical and purity specifications for food-grade bakers yeast mannoprotein, including specifications for ash, nitrogen (as a measure of protein content), carbohydrates, fraction of mannose in carbohydrates, heavy metals, and microbes. DSM provides results of the compositional analysis of four batches of bakers yeast mannoprotein meeting DSM's food-grade product specifications. DSM further notes that its specifications are consistent with those established by the Organisation Internationale du Vin, Resolution OENO 26/2004.

Bakers yeast mannoprotein is intended for use in all types of wines, including white, rosé, red, and sparkling wines, at levels ranging from 50–400 mg/L, with addition before or after the final filtration of the wine but prior

to bottling. The amount to be added is determined by the winemaker and depends on, among other factors, the amount of tartaric acid and mannoprotein naturally present in the wine.

DSM calculates the current estimated daily intake (EDI) of mannoprotein in foods taking into consideration the levels of mannoprotein naturally present in wine, beer, breads and pastries, yeast extract spreads, and dietary supplements. Using the combined results of USDA's 1994 and 1995 Continuing Survey of Food Intake by Individuals and 1994 and 1995 Diet and Health Knowledge, DSM reports a background EDI of mannoprotein of 3.6 grams per person per day (g/p/d) from these foods. DSM calculates a maximum additional EDI of 0.02 g/p/d mannoprotein from the intended addition of bakers yeast mannoprotein to wines.

As part of its safety evaluation, DSM describes published data on the natural occurrence of bakers yeast mannoprotein in a variety of foods, including wine, beer, breads and pastries, and yeast extract spreads (e.g., marmite). The notifier discusses published scientific literature establishing the safety of bakers yeast in food and food ingredient production. DSM uses published literature and nuclear magnetic resonance analysis to show that the carbohydrate moiety of bakers yeast mannoprotein is substantially equivalent to naturally occurring mannoprotein in *S. cerevisiae* and in commercially-available food products manufactured with *S. cerevisiae.*

DSM states that the toxicity of bakers yeast mannoprotein has not been specifically evaluated in animal safety studies. However, the notifier discusses the results of unpublished safety studies of its bakers yeast extract, which is used as the starting material for the manufacture of bakers yeast mannoprotein. No safety concerns were raised in acute toxicity studies in mice, rats, guinea pigs, and rabbits; subacute toxicity studies in rats; or genotoxicity studies conducted on *Salmonella typhimurium*, *Escherichia coli*, and mice.

Mannoprotein is naturally occurring in wine due to the alcohol fermentation with yeast in the wine manufacturing process. The notifier conducted a literature search and states that no reports were found to date addressing allergenic or other negative effects due to oral intake of mannoprotein via wine or other sources like bread, beer and yeast. On the basis of this literature review, DSM concludes that it is unlikely the bakers yeast mannoprotein will result in sensitization or allergies due to consumption through wine.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FFDCA)

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the Federal Food Drug and Cosmetic Act (FFDCA) to, among other things, add section 301(ll). Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of DSM's notice that bakers yeast mannoprotein is GRAS for use as a stabilizing agent in wines to prevent tartaric acid precipitation, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing bakers yeast mannoprotein. Accordingly, this response should not be construed to be a statement that foods that contain bakers yeast mannoprotein, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by DSM, as well as other information available to FDA, the agency has no questions at this time regarding DSM's conclusion that bakers yeast mannoprotein is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of bakers yeast mannoprotein. As always, it is the continuing responsibility of DSM to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000284, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying via the FDA home page at http://www.fda.gov. To view or obtain an electronic copy of the text of the letter, follow the hyperlinks from the "Food" topic to the "Food Ingredients and Packaging" section to the "Generally Recognized as Safe (GRAS)" page where the GRAS Inventory is listed.

Sincerely,

Mitchell A. Cheeseman, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Page Last Updated: 09/15/2009

Note: If you need help accessing information in different file formats, see Instructions for Downloading Viewers and Players.

U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993
Ph. 1-888-INFO-FDA (1-888-463-6332)
Email FDA

Food

Agency Response Letter GRAS Notice No. GRN 000350

CFSAN/Office of Food Additive Safety

February 4, 2011

John Husnik, Ph.D.
Phyterra Yeast Inc.
P.O. Box 21147
Charlottetown, PEI, C1A 9H6
CANADA

Re: GRAS Notice No. GRN 000350

Dear Dr. Husnik:

The Food and Drug Administration (FDA) is responding to the notice, dated August 9, 2010, that Phyterra Yeast Inc. (Phyterra) submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on August 11, 2010, filed it on August 19, 2010, and designated it as GRAS Notice No. GRN 000350.

The subject of the notice is *Saccharomyces cerevisiae* strain P1Y0, a variant of *S. cerevisiae* parent strain UCD2034, which is commonly used in the wine industry. The notice informs FDA of the view of Phyterra that *S. cerevisiae* strain P1Y0 is GRAS, through scientific procedures, for use as a yeast starter culture for alcoholic beverage fermentation such as grape must, brewing wort, and rice fermentations. The *S. cerevisiae* strain P1Y0 may be used in the reduction of hydrogen sulfide (H_2S) concentration during the production of red and white wine, champagne, sherry, sake and other rice wines, and beer. Phyterra recommends using 0.2 grams of active dry yeast per liter of wine for grape must fermentation.

In an electronic mail to Phyterra dated February 2, 2011, FDA requested clarification regarding the applicability of Phyterra's discussion of the wine-making process to alcoholic beverage fermentation in general. In an amendment dated February 3, 2011, Phyterra stated the following: "For each of the various products, bio-conversion of sugar to alcohol by yeast is the same. The raw substrate for providing the sugar may be different (grain, fruit or vegetable) and some of the processing steps to develop the individual characteristics of the products may also differ; however, our technology is applied to the yeast that is completing the bio-conversion of sugar to alcohol without hydrogen sulfide being produced - as described in the submission."

Phyterra notes that the presence of H_2S, a by-product of fermentation processes, is considered undesirable because of its odor. H_2S forms other reactive compounds, such as mercaptans and thiols that further add to the undesirable odors and flavors of the product. The purpose of developing *S. cerevisiae* strain P1Y0 is to replace the *MET10* allele of strain UCD2034 with a "low H_2S" *MET10* allele, to result in significant reduction in the production of H_2S by the yeast.[1]

Phyterra describes published and unpublished information about the development of *S. cerevisiae* strain P1Y0 and the use of *S. cerevisiae* in general. Phyterra notes that *S. cerevisiae* is a human commensal organism, with an extensive history of safe use in the production of food and beverages, including alcoholic beverages. *S. cerevisiae* strain P1Y0 strain contains an allele of the *MET10* gene from *S. cerevisiae* strain UCD932. The notifier refers to the *MET10* allele from strain UCD932 as the "low H_2S" *MET10* allele. After replacement of the native MET 10 gene of strain UCD2034 with the "low H_2S" *MET10* allele, the modified strain was designated P1Y0. The "low H_2S" *MET10* allele differs from the native *MET10* allele by only three nucleotides. This three-nucleotide difference results: (1) in two amino acid changes in the enzyme subunit it encodes and (2) in the enhancement of the sulfate assimilation pathway.

In the notice, Phyterra describes the process of introducing the "low H_2S" *MET10* allele into *S. cerevisiae* UCD2034 strain to create *S. cerevisiae* P1Y0 strain. Phyterra also describes the molecular characterization and stability assessment of the "low H_2S" *MET10* allele in *S. cerevisiae* strain P1Y0. Phyterra describes the construction of the recombinant yeast strain as "self-cloned," i.e., the final strain designated P1Y0 contains genetic material exclusively from *S. cerevisiae*. Phyterra also states that the "low H_2S" *MET10* allele, which encodes the subunit alpha of assimilatory sulfite reductase in *S. cerevisiae* strain P1Y0, is not toxic or allergenic; and is not implicated in the formation of undesirable compounds.

Phyterra describes the method of manufacture for *S. cerevisiae* strain P1Y0. *S. cerevisiae* strain P1Y0 is grown on

cane sugar and molasses under highly aerobic conditions to assure good survival during fermentation. The strain is harvested via centrifugation, filtered, and the resultant mass is extruded and dried to obtain the final product. Phyterra notes that the method of manufacture is based on well-established procedures for the production of active dry yeast used in baking, brewing, or winemaking and is in accordance with good manufacturing practice.

Phyterra considers the potential dietary exposure to *S. cerevisiae* strain P1Y0 to be negligible because the processing procedures used in winemaking remove yeast cells, debris associated with autolysed yeast cells, and most protein released during autolysis of yeast cells.

Phyterra describes generally available information on winemaking procedures and states that use of *S. cerevisiae* strain P1Y0 does not lead to any changes in the procedures or in the composition of the wine with the exception of a lower H_2S concentration. Phyterra states that the presence of the "low H_2S" *MET10* protein would occur only if the wine has been stored on lees after alcoholic fermentation. However, clarification processes, which are obligatory winemaking practices, will remove part of the *MET10* protein, as well as larger polypeptide fragments of this protein. Hence, only the smaller polypeptides and amino acids of these proteins will remain in the wine. Phyterra further notes that the use of S. cerevisiae strain P1Y0, as compared to strain UCD2034, will not result in a significant difference in the overall amino acid profiles or peptide profiles in the final product.

Phyterra describes generally available information on the clarification of wine. During wine clarification, solid particles may be allowed to settle by gravity or centrifugation. Phyterra states that clarification removes most of the yeast cells. When the wine is kept on lees for ageing, the yeast cells undergo autolysis, releasing cellular materials that are degraded through the action of enzymes. Further, white wines can be treated with bentonite to remove all remaining protein. In red wines, tannins associate with proteins; this protein-tannin complex could be precipitated by adding gelatin or egg white albumin. Phyterra states that prior to bottling, most wines undergo filtration (e.g., with diatomaceous earth, cellulose filters, or membrane filters) that can also eliminate any remaining yeast cells. Phyterra concludes that use of *S. cerevisiae* strain P1Y0 in wine is generally recognized as safe and that such wines would be comparable to wines produced using other yeast strains.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FFDCA)

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FFDCA to, among other things, add section 301(ll). Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Phyterra's notice that *S. cerevisiae* strain P1Y0 is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing *S. cerevisiae* strain P1Y0. Accordingly, this response should not be construed to be a statement that foods that contain *S. cerevisiae* strain P1Y0, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Phyterra, as well as other information available to FDA, the agency has no questions at this time regarding Phyterra's conclusion that *S. cerevisiae* strain P1Y0 is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of *S. cerevisiae* strain P1Y0. As always, it is the continuing responsibility of Phyterra to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000350, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

Mitchell A. Cheeseman, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

[1]Copper has traditionally been added to wine to remove sulfur compounds. Phyterra notes that adding *S. cerevisiae* strain P1Y0 to wine will achieve the same result.

Page Last Updated: 03/10/2011
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Food

Agency Response Letter GRAS Notice No. GRN 000353

CFSAN/Office of Food Additive Safety

March 1, 2011

George A. Burdock, Ph.D.
Burdock Group
801 N. Orange Avenue
Suite 710
Orlando, FL 32801

Re: GRAS Notice No. GRN 000353

Dear Dr. Burdock:

The Food and Drug Administration (FDA) is responding to the notice, dated July 14, 2010, that you submitted on behalf of Alltech in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on September 1, 2010, filed it on September 2, 2010, and designated it as GRAS Notice No. GRN 000353.

The subject of the notice is high-selenium yeast. The notice informs FDA of the view of Alltech that high-selenium yeast is GRAS, through scientific procedures, for use as an ingredient in yogurts, breads, instant cereals, breakfast and granola-type bars, soups, beverages, pastas, crackers, salty snacks, pretzels, and popcorn at levels of 5 to 60 milligrams per kilogram (mg/kg); and, in medical foods such that the daily intake will not exceed 19.2 mg per day (mg/day).

Our use of "high-selenium yeast" in this letter should not be considered an endorsement or recommendation of that term as an appropriate common or usual name for the purpose of declaring the substance in the ingredient statement of foods that contain that ingredient. Title 21 CFR 101.4 states that all ingredients must be declared by their common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. Issues associated with labeling and the appropriate common or usual name of a food are the responsibility of the Office of Nutrition, Labeling, and Dietary Supplements (ONLDS) in the Center for Food Safety and Applied Nutrition.

As part of its notice, Alltech includes the report of a panel of individuals (Alltech's GRAS panel) who evaluated the data and information that are the basis for Alltech's GRAS determination. Alltech considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Alltech's GRAS panel evaluated information relevant to the identity and composition of high-selenium yeast; method of manufacture and specifications for the high-selenium yeast product; estimated daily intakes of high-selenium yeast and selenium; safety studies for high-selenium yeast, and observations of the effects of selenium on human health. Based on this review, Alltech's GRAS panel concluded that high-selenium yeast is GRAS for its intended uses.

Alltech discusses the identity and composition of high-selenium yeast. Alltech describes the product as a dried (non-viable) baker's yeast, *Saccharomyces cerevisiae* strain CNCM I-3060. Alltech states that the product consists of dried yeast containing selenoproteins which incorporate selenomethionine and selenocysteine. The product also contains residual food-grade fermentation medium.

Alltech describes the method of manufacture for the high-selenium yeast product. Alltech states that *S. cerevisiae* strain CNCM I-3060 is cultivated in a selenium-enriched fermentation medium and the fermentation process follows a typical batch-fed operation that is commonly used to produce baker's yeast. Alltech states that following fermentation, the high-selenium yeast biomass is harvested and dried.

Alltech provides specifications for the high-selenium yeast product. Alltech states that the specifications include a content of ≥2000 mg of total selenium/kg of high-selenium yeast, of which ≥98 percent is organic selenium (65 percent selenomethionine, 33 percent other organic selenium). Alltech's specifications also include limits for arsenic, lead, cadmium, and mercury, as well as microbiological specifications.

Alltech calculates the estimated daily intake (EDI) of high-selenium yeast and selenium for the U.S. population using the intended food categories and use levels described in GRN 000353 and food consumption data from the USDA's National Health and Nutrition Examination Survey/What We Eat in America (NHANES/WWEIA 2003-2004).

The mean and 90th percentile consumption of high-selenium yeast per person are 8.8 and 17.4 mg/day, respectively, which results in a mean and 90th percentile consumption of selenium of 17.6 and 34.8 microgram per day (µg/day). The notifier calculates a cumulative EDI for selenium from the intended uses of high-selenium yeast and dietary selenium of 123.3 and 246.2 µg/day at the mean and 90th percentile, respectively. The notifier concludes that medical food products may contain high-selenium yeast such that the daily intake will not exceed 19.2 mg/day. Alltech states that medical foods containing high-selenium yeast are intended to be used by a select population of individuals who are under the supervision of a physician, and that these medical foods are typically formulated to replace one or more meals throughout the day. Alltech calculates a mean and 90th percentile consumption of selenium from conventional foods, intended uses of high-selenium yeast, and dietary supplements of 323.2 and 446.2 µg/day, respectively (assuming that the dietary supplements contain 200 µg selenium). Alltech states that in the unlikely event that an individual consumes conventional foods enriched with high-selenium yeast at 90th percentile levels, medical foods containing high-selenium yeast, and dietary supplements containing 200 µg selenium on a daily basis, the 90th percentile consumption of selenium would be 484.6 µg/day. Alltech states that this level is higher than the upper limit of 400 µg/day selenium established by the Institute of Medicine, but lower than the No Observed Adverse Effect Level for clinical selenosis of 800 µg/day selenium.

Alltech discusses the safety of high-selenium yeast. Alltech summarizes published animal studies that investigated the potential acute and subchronic toxicity, reproductive and developmental toxicity, inhalation toxicity, and genotoxicity of high-selenium yeast. Based on the results of these studies, the authors conclude that high-selenium yeast is not toxic or genotoxic.

Alltech also discusses published studies that describe the effects of selenium on human health. Alltech discusses a human clinical study in which healthy men and women received 600 µg/day of selenium for 12 weeks. Alltech also discusses a human study in which participants received 800 µg/day of selenium for 16 weeks. The results of both studies showed no adverse treatment-related effects.

Alltech states that the results of the published studies discussed above support the safety of addition of 200 µg selenium/day (approximately 3.3 µg selenium/kg bw/day) to the human diet.

FDA notes that some of the human health studies cited in GRN 000353 are also cited in GRN 000260, the subject of which was high-selenium yeast. In GRN 000260, the notifier explicitly discussed and dismissed concerns regarding selenium and diabetes; Alltech discusses and dismisses these concerns in GRN 000353. FDA also notes that in GRN 000353, Alltech discusses studies that were published after FDA responded to GRN 000260. These studies in humans address potential effects of selenium consumption on human health. Alltech indicates that the results of these studies do not raise concerns regarding the safety of high-selenium yeast for the intended uses.

Standards of Identity

In the notice, Alltech states its intention to use high-selenium yeast in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Potential Labeling Issues

In describing the biological effects of high-selenium yeast in human clinical studies as part of the information that Alltech relies on to conclude that high-selenium yeast is GRAS under the conditions of its intended use, Alltech raises a potential issue under the labeling provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA). Under section 403(a) of the FFDCA, a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FFDCA lays out the statutory framework for the use of labeling claims that characterize the level of a nutrient in a food or that characterize the relationship of a nutrient to a disease or health-related condition. If products that contain high-selenium yeast bear any claims on the label or in labeling, such claims are the purview of ONLDS. The Office of Food Additive Safety neither consulted with ONLDS on this labeling issue nor evaluated the information in your notice to determine whether it would support any claims made about high-selenium yeast on the label or in labeling.

Medical Foods

In its notice, Alltech informs FDA that one intended use of high-selenium yeast is use in medical foods. Section 5 (b) of the Orphan Drug Act (ODA) defines a medical food as a food that is formulated to be consumed or administered enterally under the supervision of a physician and that is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation. Section 403(q) of the FFDCA lays out the statutory framework for nutrition labeling of food products. Section 403(r) of the FFDCA lays out the statutory framework for health claims and nutrient content claims. Under section 403(q)(5)(A)(iv) of the FFDCA and FDA's implementing regulations in 21 CFR 101.9(j)(8), the requirements for nutrition labeling do not apply to medical foods as defined in section 5(b) of the ODA. Under section 403(r)(5)(A) of the FFDCA and FDA's implementing regulations in 21

CFR 101.13(q)(4)(ii) and 21 CFR 101.14(f)(2), the requirements for nutrient content claims and health claims, respectively, do not apply to medical foods as defined in section 5(b) of the ODA. For your information, FDA's response to Alltech's notice that high-selenium yeast is GRAS for use in medical foods does not address the question of whether any particular food product that contains high-selenium yeast as an ingredient would be a medical food within the meaning of section 5(b) of the ODA and, thus, would be exempt from the requirements for nutrition labeling, nutrient content claims, and health claims.

Section 301(ll) of the FFDCA

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FFDCA to, among other things, add section 301(ll). Section 301(ll) of the FFDCA prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FFDCA, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Alltech's notice that high-selenium yeast is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing high-selenium yeast. Accordingly, this response should not be construed to be a statement that foods that contain high-selenium yeast, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Alltech, as well as other information available to FDA, the agency has no questions at this time regarding Alltech's conclusion that high-selenium yeast is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of high-selenium yeast. As always, it is the continuing responsibility of Alltech to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000353, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

Mitchell A. Cheeseman, Ph.D.
Acting Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Page Last Updated: 04/15/2011
Note: If you need help accessing information in different file formats, see Instructions for Downloading Viewers and Players.

Food

Agency Response Letter GRAS Notice No. GRN 000422

CFSAN/Office of Food Additive Safety

August 31, 2012

John Husnik, Ph.D.
Functional Technologies Corporation
Suite 1500 - 885 West Georgia Street
Vancouver, British Columbia, V6C 3E8
CANADA

Re: GRAS Notice No. GRN 000422

Dear Dr. Husnik:

The Food and Drug Administration (FDA) is responding to the notice, dated January 31, 2012, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on February 29, 2012, filed it on March 5, 2012, and designated it as GRAS Notice No. GRN 000422.

The subject of the notice is modified baker's yeast *Saccharomyces cerevisiae* (modified baker's yeast). The notice informs FDA of the view of Functional Technologies Corporation (FTC) that modified baker's yeast is GRAS, through scientific procedures, for use to reduce acrylamide production in a variety of grain-based foods, vegetable-based food (potato), and coffee and coffee substitutes at a level of 0.5 to 5%.

FTC discusses the formation and effect of acrylamide in food. Acrylamide is produced during the cooking or processing of certain foods, particularly in foods high in carbohydrates and low in proteins. Acrylamide is predominately formed from free amino acids, mainly asparagine, and reducing sugars via the complex Maillard reaction. FTC intends to use modified baker's yeast *S. cerevisiae* to prevent acrylamide formation in the notified food and beverage products.

FTC discusses the natural occurrence of *S. cerevisiae* and its use in foods. *S. cerevisiae* is a commensal organism that is normally found on the human body, in the air, and on many foods consumed by humans. *S. cerevisiae* strains have been used in the production of baked goods, wine, and beer for more than five thousand years. FTC states that the host strain used to develop modified baker's yeast is an industrial *S. cerevisiae* strain that is widely used in the baking industry. The host strain was obtained directly as a commercial culture from a product manufacturer.

FTC discusses the development and characterization of modified baker's yeast. The host strain was transformed with three copies of the *S. cerevisiae* ASP3 gene encoding for asparaginase. The resulting modified baker's yeast overexpresses asparaginase. Sequencing analyses also confirmed that the inserted DNA sequences in modified baker's yeast were all obtained from *S. cerevisiae* and that no foreign genetic material was incorporated into the yeast genome.

FTC describes the method of manufacture of modified baker's yeast to be identical to that of commercial fresh or active dry yeast from *S. cerevisiae* described in the published literature. Pure yeast inoculum is grown initially as laboratory cultures under anaerobic conditions. Subsequent steps include aerobic fermentation in the presence of essential nutrients including molasses. The fermentation harvest is centrifuged to obtain yeast cream, followed by vacuum filtration. The filtered product is extruded and dried to obtain modified baker's yeast. FTC notes that the modified baker's yeast is produced under good manufacturing practice and using food grade materials.

FTC considers the intended use of modified baker's yeast to be substitutional for typical baker's yeast currently used in the baking, snack food, and other food industries. Therefore, the dietary exposure to modified baker's yeast would be similar to that of typical baker's yeast currently in use.

Standards of Identity

In the notice, FTC states its intention to use modified baker's yeast in several food categories, including foods for which standards of identity exist, located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FD&C Act)

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FD&C Act to, among other things, add section 301(ll). Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of FTC's notice that modified baker's yeast is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing modified baker's yeast. Accordingly, this response should not be construed to be a statement that foods that contain modified baker's yeast, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by FTC, as well as other information available to FDA, the agency has no questions at this time regarding FTC's conclusion that modified baker's yeast is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of modified baker's yeast. As always, it is the continuing responsibility of FTC to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000422, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at www.fda.gov/grasnoticeinventory.

Sincerely,

Dennis M. Keefe, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

Page Last Updated: 09/21/2012
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WATER IMPLICATIONS OF BIOFUELS PRODUCTION IN THE UNITED STATES

Committee on Water Implications of Biofuels Production in the United States

Water Science and Technology Board

Division on Earth and Life Studies

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Support for this study was provided by the McKnight Foundation under Grant No. 06-1201, Energy Foundation under Grant No. G-0611-08651, National Science Foundation under Grant No. CBET-0716901, U.S. Environmental Protection Agency under Order No. EP07C000287, and the National Research Council Day Fund. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the organizations or agencies that provided support for the project.

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tions of an ethanol plant are shown in Figure 5-1. Pure water is required for the slurry operation with whole corn, followed by liquefaction to liberate sugars from starch via hydrolysis. This is followed by fermentation and distillation operations.

Current estimates of the consumptive water use from these facilities are in the range of 4 gallons of water per gallon of ethanol produced (gal/gal) (Pate et al., 2007). For perspective, consumptive water use in petroleum refining is about 1.5 gal/gal (Pate et al., 2007). Overall water use in biorefineries may be as high as 7 gal/gal, but this number has been consistently decreasing over time and as of 2005 was only slightly over 4 gal/gal in 2005 (Phillips et al., 2007). Thus for a 100 million gallon per year plant, a little over 400 million gallons of water per year would be withdrawn from aquifers or surface water sources (1.1 million gallons per day). The overall water balance for a typical bioethanol plant using corn is shown in Figure

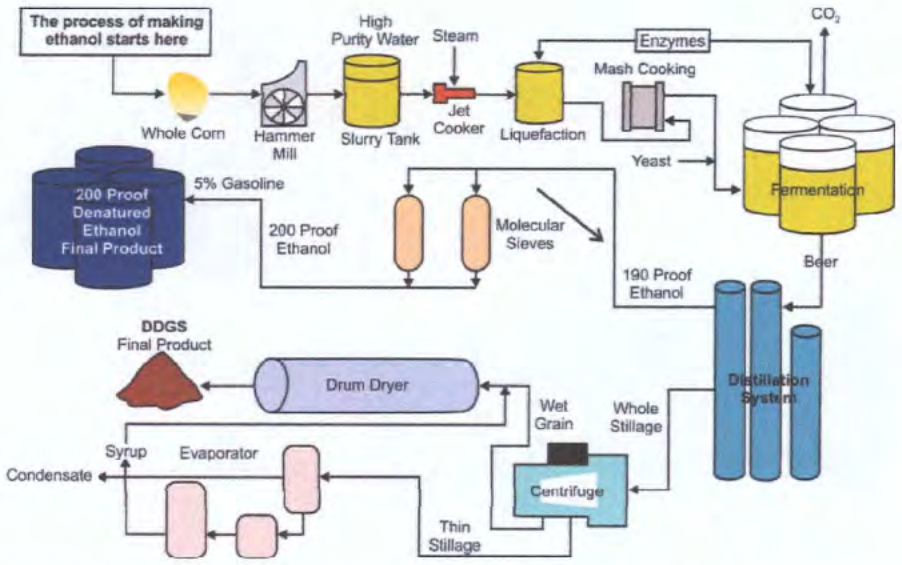

FIGURE 5-1 Process schematic and unit operations of ethanol production facility from whole corn kernels. DDGS is "dry distillers grains with solubles."
SOURCE: Parkin et al. (2007).

10 pages withheld in their entirety

Pursuant FOIA exemption (b)(4) and

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NOTICE PERTINENT TO THE MARCH 2013 REVISIONS OF THE *NIH GUIDELINES FOR RESEARCH INVOLVING RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES (NIH GUIDELINES)*

Summary of Amendments:
Major Actions

Page 1: Title changed from "*NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines)*" to "*NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines)*"

Page 10: **Section I-A. Purpose.** Added the new numbered clause:
"(ii) synthetic nucleic acid molecules, including those that are chemically or otherwise modified but can base pair with naturally occurring nucleic acid molecules"

Page 10: **Section I-B. Definition...** Revised definition:
"(i) molecules that a) are constructed by joining nucleic acid molecules and b) that can replicate in a living cell, i.e. recombinant nucleic acids;
(ii) nucleic acid molecules that are chemically or by other means synthesized or amplified, including those that are chemically or otherwise modified but can base pair with naturally occurring nucleic acid molecules, i.e. synthetic nucleic acids, [...]"

Deleted the following paragraphs:
"Synthetic DNA segments which are likely to yield a potentially harmful polynucleotide or polypeptide (e.g., a toxin or a pharmacologically active agent) are considered as equivalent to their natural DNA counterpart. If the synthetic DNA segment is not expressed *in vivo* as a biologically active polynucleotide or polypeptide product, it is exempt from the *NIH Guidelines*."

"Genomic DNA of plants and bacteria that have acquired a transposable element, even if the latter was donated from a recombinant vector no longer present, are not subject to the *NIH Guidelines* unless the transposon itself contains recombinant DNA."

Page 13: **Section II-A-3. Comprehensive [...]** Additions:
First paragraph: "[...] the first step is to assess the risk of the agent itself. Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, classifies agents into Risk Groups based on an assessment of their ability to cause disease in humans and the available treatments for such disease. Once the Risk Group of the agent is identified, this should be followed by [...].

New paragraphs (2) and (3):
"While the starting point for the risk assessment is based on the identification of the Risk Group of the parent agent, as technology moves forward, it may be possible to develop an organism containing genetic sequences from multiple sources such that the parent agent may not be obvious. In such cases, the risk assessment should include at least two levels of analysis. The first involves a consideration of the Risk Groups of the source(s) of the sequences and the second involves an assessment of the functions that may be encoded by these sequences (e.g., virulence or transmissibility). It may be prudent to first consider the highest Risk Group classification of all agents that are the source of sequences included in the construct. Other factors to be considered include the percentage of the genome contributed by each parent agent and the predicted function or intended purpose of each contributing sequence. The initial assumption should be that all sequences will function as they did in the original host context."

"The Principal Investigator and Institutional Biosafety Committee must also be cognizant that the combination of certain sequences in a new biological context may result in an organism whose risk profile could be higher than that of the contributing organisms or sequences. The synergistic function of these sequences may be one of the key attributes to consider in deciding whether a higher containment level is warranted, at least until further assessments can be carried out. A new biosafety risk may occur with an organism formed through combination of sequences from a number of organisms or due to the synergistic effect of combining transgenes that results in a new phenotype." [...]

Page 16: **Section III-A-1-a.** New paragraphs (2) and (3):
"Consideration should be given as to whether the drug resistance trait to be used in the experiment would render that microorganism resistant to the primary drug available to and/or indicated for certain populations, for example children or pregnant women."

"At the request of an Institutional Biosafety Committee, NIH/OBA will make a determination regarding whether a specific experiment involving the deliberate transfer of a drug resistance trait falls under Section III-A-1-a and therefore requires RAC review and NIH Director approval. An Institutional Biosafety Committee may also consult with NIH/OBA regarding experiments that do not meet the requirements of Section III-A-1-a but nonetheless raise important public health issues. NIH/OBA will consult, as needed, with one or more experts, which may include the RAC."

Section III-B-2. New Section:
Experiments that have been Approved (under Section III-A-1-a) as Major Actions under the *NIH Guidelines*

Upon receipt and review of an application from the investigator, NIH/OBA may determine that a proposed experiment is equivalent to an experiment that has previously been approved by the NIH Director as a Major Action, including experiments approved prior to implementation of these changes. An experiment will only be considered equivalent if, as determined by NIH/OBA, there are no substantive differences and pertinent information has not emerged since submission of the initial III-A-1-a experiment that would change the biosafety and public health considerations for the proposed experiments. If such a determination is made by NIH/OBA, these experiments will not require review and approval under Section III-A.

Page 17: **Section III-C-1.** Revised definition:
"[...]Human gene transfer is the deliberate transfer into human research participants of either:

1. Recombinant nucleic acid molecules, or DNA or RNA derived from recombinant nucleic acid molecules, or
2. Synthetic nucleic acid molecules or DNA, or RNA derived from synthetic nucleic acid molecules, that meet any one of the following criteria:
    a. Contain more than 100 nucleotides; or
    b. Possess biological properties that enable integration into the genome (e.g., *cis* elements involved in integration); or
    c. Have the potential to replicate in a cell; or
    d. Can be translated or transcribed."

Page 23: **Section III-F. Exempt Experiments** Added:
"[...] however, other federal and state standards of biosafety may still apply to such research (for example, the Centers for Disease Control and Prevention (CDC)/NIH publication Biosafety in Microbiological and Biomedical Laboratories)."

Page 23: **Section III-F-1.** New section:
"Those synthetic nucleic acids that: (1) can neither replicate nor generate nucleic acids that can replicate in any living cell (e.g., oligonucleotides or other synthetic nucleic acids that

do not contain an origin of replication or contain elements known to interact with either DNA or RNA polymerase), and (2) are not designed to integrate into DNA, and (3) do not produce a toxin that is lethal for vertebrates at an LD50 of less than 100 nanograms per kilogram body weight. If a synthetic nucleic acid is deliberately transferred into one or more human research participants and meets the criteria of Section III-C, it is not exempt under this Section."

Page 23: **Section III-F-2.** Updated section:
"Those that are not in organisms, cells or viruses and that have not been modified or manipulated (e.g., encapsulated into synthetic or natural vehicles) to render them capable of penetrating cellular membranes."

Page 23: **Section III-F-7.** New Section (in part moved from prior version of Section I-B – see deleted paragraphs above):
"Those genomic DNA molecules that have acquired a transposable element, provided the transposable element does not contain any recombinant and/or synthetic DNA."

Pages 23-24: Renumbered: Sections III-F-1 through III-F-8

Page 24: **Section IV-A. Policy.** Paragraph 1, added:
"[…]The utilization of new genetic manipulation techniques may enable work previously conducted using recombinant means to be accomplished faster, more efficiently, or at larger scale. These techniques have not yet yielded organisms that present safety concerns that fall outside the current risk assessment framework used for recombinant nucleic acid research. Nonetheless, an appropriate risk assessment of experiments involving these techniques must be conducted taking into account the way these approaches may alter the risk assessment. As new techniques develop, the *NIH Guidelines* should be periodically reviewed to determine whether and how such research should be explicitly addressed."

New Paragraph 2, deleted the sentence:
"General recognition of institutional authority and responsibility properly establishes accountability for safe conduct of the research at the local level."

Throughout: The term "recombinant" in a variety of contexts (e.g., recombinant DNA, recombinant molecules, recombinant techniques…) has been replaced with the terms "recombinant or synthetic" as appropriate.

Minor Actions:

Page 39: **Appendix B-I:** All serotypes of Adenovirus-Associated Viruses are considered to be Risk Group 1 agents. Previously, only serotypes 1 – 4 were listed.

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VOLUME

NIH GUIDELINES FOR RESEARCH INVOLVING RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES (NIH GUIDELINES)

March 2013

DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

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Visit the OBA Web site at:
http://oba.od.nih.gov
For current information on Guidelines, Protocols, Principal Investigators, Meetings, and information about upcoming Gene Therapy Policy Conferences

These *NIH Guidelines* shall be in effect on March 5, 2013 and on that date, shall supersede all earlier versions until further notice.

****

FEDERAL REGISTER NOTICES

Effective June 24, 1994, Published in Federal Register, July 5, 1994 (59 FR 34472)
Amendment Effective July 28, 1994, Federal Register, August 5, 1994 (59 FR 40170)
Amendment Effective April 17, 1995, Federal Register, April 27, 1995 (60 FR 20726)
Amendment Effective December 14, 1995, Federal Register, January 19, 1996 (61 FR 1482)
Amendment Effective March 1, 1996, Federal Register, March 12, 1996 (61 FR 10004)
Amendment Effective January 23, 1997, Federal Register, January 31, 1997 (62 FR 4782)
Amendment Effective September 30, 1997, Federal Register, October 14, 1997 (62 FR 53335)
Amendment Effective October 20, 1997, Federal Register, October 29, 1997 (62 FR 56196)
Amendment Effective October 22, 1997, Federal Register, October 31, 1997 (62 FR 59032)
Amendment Effective February 4, 1998, Federal Register, February 17, 1998 (63 FR 8052)
Amendment Effective April 30, 1998, Federal Register, May 11, 1998 (63 FR 26018)
Amendment Effective April 29, 1999, Federal Register, May 11, 1999 (64 FR 25361)
Amendment Effective October 2, 2000, Federal Register, October 10, 2000 (65 FR 60328)
Amendment Effective December 28, 2000 Federal Register, January 5, 2001 (66 FR 1146)
Amendment Effective December 11, 2001 Federal Register, December 11, 2001 (66 FR 64051)
Amendment Effective December 19, 2001 Federal Register, November 19, 2001 (66 FR 57970)
Amendment Effective January 10, 2002 Federal Register, December 11, 2001 (66 FR 64052)
Amendment Effective January 24, 2002 Federal Register, November 19, 2001 (66 FR 57970)
Amendment Effective September 22, 2009 Federal Register, September 22, 2009 (74 FR 48275)
Amendment Effective January 19, 2011 Federal Register, January 19, 2011 (76 FR 3150)
Amendment Effective May 12, 2011 Federal Register, May 12, 2011 (76 FR 27653)
Amendment Effective October 11, 2011 Federal Register, October 11, 2011 (76 FR 62816)
Amendment Effective March 5, 2013 Federal Register, September 5, 2012 (77 FR 54584)

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SECTION I. SCOPE OF THE *NIH GUIDELINES*

Section I-A. Purpose

The purpose of the *NIH Guidelines* is to specify the practices for constructing and handling: (i) recombinant nucleic acid molecules, (ii) synthetic nucleic acid molecules, including those that are chemically or otherwise modified but can base pair with naturally occurring nucleic acid molecules, and (iii) cells, organisms, and viruses containing such molecules.

Section I-A-1. Any nucleic acid molecule experiment, which according to the *NIH Guidelines* requires approval by NIH, must be submitted to NIH or to another Federal agency that has jurisdiction for review and approval. Once approvals, or other applicable clearances, have been obtained from a Federal agency other than NIH (whether the experiment is referred to that agency by NIH or sent directly there by the submitter), the experiment may proceed without the necessity for NIH review or approval. (See exception in Section I-A-1-a regarding requirement for human gene transfer protocol registration.)

Section I-A-1-a. For experiments involving the deliberate transfer of recombinant or synthetic nucleic acid molecules, or DNA or RNA derived from recombinant or synthetic nucleic acid molecules, into human research participants (human gene transfer), no research participant shall be enrolled (see definition of enrollment in Section I-E-7) until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*); Institutional Biosafety Committee (IBC) approval (from the clinical trial site) has been obtained; Institutional Review Board approval has been obtained; and all applicable regulatory authorization(s) have been obtained.

For a clinical trial site that is added after the RAC review process, no research participant shall be enrolled (see definition of enrollment in Section I-E-7) at the clinical trial site until the following documentation has been submitted to NIH OBA: (1) IBC approval (from the clinical trial site); (2) Institutional Review Board approval; (3) Institutional Review Board-approved informed consent document; and (4) curriculum vitae of the Principal Investigator(s) (no more than two pages in biographical sketch format); and (5) NIH grant number(s) if applicable.

Section I-B. Definition of Recombinant and Synthetic Nucleic Acid Molecules

In the context of the *NIH Guidelines*, recombinant and synthetic nucleic acids are defined as:

(i) molecules that a) are constructed by joining nucleic acid molecules and b) that can replicate in a living cell, i.e., recombinant nucleic acids;
(ii) nucleic acid molecules that are chemically or by other means synthesized or amplified, including those that are chemically or otherwise modified but can base pair with naturally occurring nucleic acid molecules, i.e., synthetic nucleic acids, or
(iii) molecules that result from the replication of those described in (i) or (ii) above.

Section I-C. General Applicability

Section I-C-1. The *NIH Guidelines* are applicable to:

Section I-C-1-a. All recombinant or synthetic nucleic acid research within the United States (U.S.) or its territories that is within the category of research described in either Section I-C-1-a-(1) or Section I-C-1-a-(2).

Section I-C-1-a-(1). Research that is conducted at or sponsored by an institution that receives any support for recombinant or synthetic nucleic acid research from NIH, including research performed directly by NIH. An individual who receives support for research involving recombinant or synthetic nucleic acids must be associated with or sponsored by an institution that assumes the responsibilities assigned in the *NIH Guidelines*.

Section I-C-1-a-(2). Research that involves testing in humans of materials containing recombinant or synthetic nucleic acids developed with NIH funds, if the institution that developed those materials sponsors or participates in those projects. Participation includes research collaboration or contractual agreements, not mere provision of research materials.

Section I-C-1-b. All recombinant or synthetic nucleic acid research performed abroad that is within the category of research described in either Section I-C-1-b-(1) or Section I-C-1-b-(2).

Section I-C-1-b-(1). Research supported by NIH funds.

Section I-C-1-b-(2). Research that involves testing in humans of materials containing recombinant or synthetic nucleic acids developed with NIH funds, if the institution that developed those materials sponsors or participates in those projects. Participation includes research collaboration or contractual agreements, not mere provision of research materials.

Section I-C-1-b-(3). If the host country has established rules for the conduct of recombinant or synthetic nucleic acid molecule research, then the research must be in compliance with those rules. If the host country does not have such rules, the proposed research must be reviewed and approved by an NIH-approved Institutional Biosafety Committee or equivalent review body and accepted in writing by an appropriate national governmental authority of the host country. The safety practices that are employed abroad must be reasonably consistent with the *NIH Guidelines*.

Section I-D. Compliance with the *NIH Guidelines*

As a condition for NIH funding of recombinant or synthetic nucleic acid molecule research, institutions shall ensure that such research conducted at or sponsored by the institution, irrespective of the source of funding, shall comply with the *NIH Guidelines*.

Information concerning noncompliance with the *NIH Guidelines* may be brought forward by any person. It should be delivered to both NIH/OBA and the relevant institution. The institution, generally through the Institutional Biosafety Committee, shall take appropriate action. The institution shall forward a complete report of the incident recommending any further action to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985, 301-496-9838/301-496-9839 (fax) (for non-USPS mail, use zip code 20817).

In cases where NIH proposes to suspend, limit, or terminate financial assistance because of noncompliance with the *NIH Guidelines*, applicable DHHS and Public Health Service procedures shall govern.

The policies on compliance are as follows:

Section I-D-1. All NIH-funded projects involving recombinant or synthetic nucleic acid molecule must comply with the *NIH Guidelines*. Non-compliance may result in: (i) suspension, limitation, or termination of financial assistance for the noncompliant NIH-funded research project and of NIH funds for other recombinant or synthetic nucleic acid molecule research at the institution, or (ii) a requirement for prior NIH approval of any or all recombinant or synthetic nucleic acid molecule projects at the institution.

Section I-D-2. All non-NIH funded projects involving recombinant or synthetic nucleic acid molecule conducted at or sponsored by an institution that receives NIH funds for projects involving such techniques must comply with the *NIH Guidelines*. Noncompliance may result in: (i) suspension, limitation, or termination of NIH funds for recombinant or synthetic nucleic acid molecule research at the institution, or (ii) a requirement for prior NIH approval of any or all recombinant or synthetic nucleic acid molecule projects at the institution.

Section I-E. General Definitions

The following terms, which are used throughout the *NIH Guidelines*, are defined as follows:

Section I-E-1. An "institution" is any public or private entity (including Federal, state, and local government agencies).

Section I-E-2. An "Institutional Biosafety Committee" is a committee that: (i) meets the requirements for membership specified in Section IV-B-2, *Institutional Biosafety Committee (IBC)*, and (ii) reviews, approves, and oversees projects in accordance with the responsibilities defined in Section IV-B-2, *Institutional Biosafety Committee (IBC)*.

Section I-E-3. The "Office of Biotechnology Activities (OBA)" is the office within the NIH that is responsible for: (i) reviewing and coordinating all activities relating to the *NIH Guidelines*, and (ii) performing other duties as defined in Section IV-C-3, *Office of Biotechnology Activities (OBA)*.

Section I-E-4. The "Recombinant DNA Advisory Committee" is the public advisory committee that advises the Department of Health and Human Services (DHHS) Secretary, the DHHS Assistant Secretary for Health, and the NIH Director concerning recombinant or synthetic nucleic acid molecule research. The RAC shall be constituted as specified in Section IV-C-2, *Recombinant DNA Advisory Committee (RAC)*.

Section I-E-5. The "NIH Director" is the Director of the National Institutes of Health, or any other officer or employee of NIH to whom authority has been delegated.

Section I-E-6. "Deliberate release" is defined as a planned introduction of recombinant or synthetic nucleic acid molecule-containing microorganisms, plants, or animals into the environment.

Section I-E-7. "Enrollment" is the process of obtaining informed consent from a potential research participant, or a designated legal guardian of the participant, to undergo a test or procedure associated with the gene transfer experiment.

Section I-E-8. A "serious adverse event" is any event occurring at any dose that results in any of the following outcomes: death, a life-threatening event, in-patient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization also may be considered a serious adverse event when, upon the basis of appropriate medical judgment, they may jeopardize the human gene transfer research subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

Section I-E-9. An adverse event is "associated with the use of a gene transfer product" when there is a reasonable possibility that the event may have been caused by the use of that product.

Section I-E-10. An "unexpected serious adverse event" is any serious adverse event for which the specificity or severity is not consistent with the risk information available in the current investigator's brochure.

****

SECTION II. SAFETY CONSIDERATIONS

Section II-A. Risk Assessment

Section II-A-1. Risk Groups

Risk assessment is ultimately a subjective process. The investigator must make an initial risk assessment based on the Risk Group (RG) of an agent (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*). Agents are classified into four Risk Groups (RGs) according to their relative pathogenicity for healthy adult humans by the following criteria: (1) Risk Group 1 (RG1) agents are not associated with disease in healthy adult humans. (2) Risk Group 2 (RG2) agents are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are *often* available. (3) Risk Group 3 (RG3) agents are associated with serious or lethal human disease for which preventive or therapeutic interventions *may be* available. (4) Risk Group 4 (RG4) agents are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are *not usually* available.

Section II-A-2. Criteria for Risk Groups

Classification of agents in Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, is based on the potential effect of a biological agent on a healthy human adult and does not account for instances in which an individual may have increased susceptibility to such agents, e.g., preexisting diseases, medications, compromised immunity, pregnancy or breast feeding (which may increase exposure of infants to some agents).

Personnel may need periodic medical surveillance to ascertain fitness to perform certain activities; they may also need to be offered prophylactic vaccines and boosters (see Section IV-B-1-f, *Responsibilities of the Institution, General Information*).

Section II-A-3. Comprehensive Risk Assessment

In deciding on the appropriate containment for an experiment, the first step is to assess the risk of the agent itself. Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, classifies agents into Risk Groups based on an assessment of their ability to cause disease in humans and the available treatments for such disease. Once the Risk Group of the agent is identified, this should be followed by a thorough consideration of how the agent is to be manipulated. Factors to be considered in determining the level of containment include agent factors such as: virulence, pathogenicity, infectious dose, environmental stability, route of spread, communicability, operations, quantity, availability of vaccine or treatment, and gene product effects such as toxicity, physiological activity, and allergenicity. Any strain that is known to be more hazardous than the parent (wild-type) strain should be considered for handling at a higher containment level. Certain attenuated strains or strains that have been demonstrated to have irreversibly lost known virulence factors may qualify for a reduction of the containment level compared to the Risk Group assigned to the parent strain (see Section V-B, *Footnotes and References of Sections I-IV*).

While the starting point for the risk assessment is based on the identification of the Risk Group of the parent agent, as technology moves forward, it may be possible to develop an organism containing genetic sequences from multiple sources such that the parent agent may not be obvious. In such cases, the risk assessment should include at least two levels of analysis. The first involves a consideration of the Risk Groups of the source(s) of the sequences and the second involves an assessment of the functions that may be encoded by these sequences (e.g., virulence or transmissibility). It may be prudent to first consider the highest Risk Group classification of all agents that are the source of sequences included in the construct. Other factors to be considered include the percentage of the genome contributed by each parent agent and the predicted function or intended purpose of each contributing sequence. The initial assumption should be that all sequences will function as they did in the original host context.

The Principal Investigator and Institutional Biosafety Committee must also be cognizant that the combination of certain sequences in a new biological context may result in an organism whose risk profile could be higher than that of the contributing organisms or sequences. The synergistic function of these sequences may be one of the key attributes to consider in deciding whether a higher containment level is warranted, at least until further assessments can be carried out. A new biosafety risk may occur with an organism formed through combination of sequences from a number of organisms or due to the synergistic effect of combining transgenes that results in a new phenotype.

A final assessment of risk based on these considerations is then used to set the appropriate containment conditions for the experiment (see Section II-B, *Containment*). The appropriate containment level may be equivalent to the Risk Group classification of the agent or it may be raised or lowered as a result of the above considerations. The Institutional Biosafety Committee must approve the risk assessment and the biosafety containment level for recombinant or synthetic nucleic acid experiments described in Sections III-A, *Experiments that Require Institutional Biosafety Committee Approval, RAC Review, and NIH Director Approval Before Initiation*; III-B, *Experiments that Require NIH/OBA and Institutional Biosafety Committee Approval Before Initiation*; III-C, *Experiments that Require Institutional Biosafety Committee and Institutional Review Board Approvals and NIH/OBA Registration Before Initiation*; III-D, *Experiments that Require Institutional Biosafety Committee Approval Before Initiation*.

Careful consideration should be given to the types of manipulation planned for some higher Risk Group agents. For example, the RG2 dengue viruses may be cultured under the Biosafety Level (BL) 2 containment (see Section II-B); however, when such agents are used for animal inoculation or transmission studies, a higher containment level is recommended. Similarly, RG3 agents such as Venezuelan equine encephalomyelitis and yellow fever viruses should be handled at a higher containment level for animal inoculation and transmission experiments.

Individuals working with human immunodeficiency virus (HIV), hepatitis B virus (HBV) or other bloodborne pathogens should consult the applicable Occupational Safety and Health Administration (OSHA) regulation, 29 CFR 1910.1030, and OSHA publication 3127 (1996 revised). BL2 containment is recommended for activities

involving all blood-contaminated clinical specimens, body fluids, and tissues from all humans, or from HIV- or HBV-infected or inoculated laboratory animals. Activities such as the production of research-laboratory scale quantities of HIV or other bloodborne pathogens, manipulating concentrated virus preparations, or conducting procedures that may produce droplets or aerosols, are performed in a BL2 facility using the additional practices and containment equipment recommended for BL3. Activities involving industrial scale volumes or preparations of concentrated HIV are conducted in a BL3 facility, or BL3 Large Scale if appropriate, using BL3 practices and containment equipment.

Exotic plant pathogens and animal pathogens of domestic livestock and poultry are restricted and may require special laboratory design, operation and containment features not addressed in *Biosafety in Microbiological and Biomedical Laboratories* (see Section V-C, *Footnotes and References of Sections I through IV*). For information regarding the importation, possession, or use of these agents see Sections V-G and V-H, *Footnotes and References of Sections I through IV*.

Section II-B. Containment

Effective biological safety programs have been operative in a variety of laboratories for many years. Considerable information already exists about the design of physical containment facilities and selection of laboratory procedures applicable to organisms carrying additional recombinant or synthetic nucleic acid molecules (see Section V-B, *Footnotes and References of Sections I-IV*). The existing programs rely upon mechanisms that can be divided into two categories: (i) a set of standard practices that are generally used in microbiological laboratories; and (ii) special procedures, equipment, and laboratory installations that provide physical barriers that are applied in varying degrees according to the estimated biohazard. Four biosafety levels are described in Appendix G, *Physical Containment*. These biosafety levels consist of combinations of laboratory practices and techniques, safety equipment, and laboratory facilities appropriate for the operations performed and are based on the potential hazards imposed by the agents used and for the laboratory function and activity. Biosafety Level 4 provides the most stringent containment conditions, Biosafety Level 1 the least stringent.

Experiments involving recombinant or synthetic nucleic acid molecules lend themselves to a third containment mechanism, namely, the application of highly specific biological barriers. Natural barriers exist that limit either: (i) the infectivity of a vector or vehicle (plasmid or virus) for specific hosts, or (ii) its dissemination and survival in the environment. Vectors, which provide the means for recombinant or synthetic nucleic acid molecule and/or host cell replication, can be genetically designed to decrease, by many orders of magnitude, the probability of dissemination of recombinant or synthetic nucleic acid molecule outside the laboratory (see Appendix I, *Biological Containment*).

Since these three means of containment are complementary, different levels of containment can be established that apply various combinations of the physical and biological barriers along with a constant use of standard practices. Categories of containment are considered separately in order that such combinations can be conveniently expressed in the *NIH Guidelines*.

Physical containment conditions within laboratories, described in Appendix G, *Physical Containment*, may not always be appropriate for all organisms because of their physical size, the number of organisms needed for an experiment, or the particular growth requirements of the organism. Likewise, biological containment for microorganisms described in Appendix I, *Biological Containment*, may not be appropriate for all organisms, particularly higher eukaryotic organisms. However, significant information exists about the design of research facilities and experimental procedures that are applicable to organisms containing additional recombinant or synthetic nucleic acid molecules that are either integrated into the genome or into microorganisms associated with the higher organism as a symbiont, pathogen, or other relationship. This information describes facilities for physical containment of organisms used in non-traditional laboratory settings and special practices for limiting or excluding the unwanted establishment, transfer of genetic information, and dissemination of organisms beyond the intended location, based on both physical and biological containment principles. Research conducted in accordance with these conditions effectively confines the organism.

For research involving plants, four biosafety levels (BL1-P through BL4-P) are described in Appendix P, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Plants*. BL1-P is designed to provide a moderate level of containment for experiments for which there is convincing biological evidence that precludes the possibility of survival, transfer, or dissemination of recombinant or synthetic nucleic acid molecules into the environment, or in which there is no recognizable and

predictable risk to the environment in the event of accidental release. BL2-P is designed to provide a greater level of containment for experiments involving plants and certain associated organisms in which there is a recognized possibility of survival, transmission, or dissemination of recombinant or synthetic nucleic acid molecule containing organisms, but the consequence of such an inadvertent release has a predictably minimal biological impact. BL3-P and BL4-P describe additional containment conditions for research with plants and certain pathogens and other organisms that require special containment because of their recognized potential for significant detrimental impact on managed or natural ecosystems. BL1-P relies upon accepted scientific practices for conducting research in most ordinary greenhouse or growth chamber facilities and incorporates accepted procedures for good pest control and cultural practices. BL1-P facilities and procedures provide a modified and protected environment for the propagation of plants and microorganisms associated with the plants and a degree of containment that adequately controls the potential for release of biologically viable plants, plant parts, and microorganisms associated with them. BL2-P and BL3-P rely upon accepted scientific practices for conducting research in greenhouses with organisms infecting or infesting plants in a manner that minimizes or prevents inadvertent contamination of plants within or surrounding the greenhouse. BL4-P describes facilities and practices known to provide containment of certain exotic plant pathogens.

For research involving animals, which are of a size or have growth requirements that preclude the use of conventional primary containment systems used for small laboratory animals, four biosafety levels (BL1-N through BL4-N) are described in Appendix Q, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Animals*. BL1-N describes containment for animals that have been modified by stable introduction of recombinant or synthetic nucleic acid molecules, or DNA derived therefrom, into the germ-line (transgenic animals) and experiments involving viable recombinant or synthetic nucleic acid molecule-modified microorganisms and is designed to eliminate the possibility of sexual transmission of the modified genome or transmission of recombinant or synthetic nucleic acid molecule-derived viruses known to be transmitted from animal parent to offspring only by sexual reproduction. Procedures, practices, and facilities follow classical methods of avoiding genetic exchange between animals. BL2-N describes containment which is used for transgenic animals associated with recombinant or synthetic nucleic acid molecule-derived organisms and is designed to eliminate the possibility of vertical or horizontal transmission. Procedures, practices, and facilities follow classical methods of avoiding genetic exchange between animals or controlling arthropod transmission. BL3-N
and BL4-N describe higher levels of containment for research with certain transgenic animals involving agents which pose recognized hazard.

In constructing the *NIH Guidelines*, it was necessary to define boundary conditions for the different levels of physical and biological containment and for the classes of experiments to which they apply. These definitions do not take into account all existing and anticipated information on special procedures that will allow particular experiments to be conducted under different conditions than indicated here without affecting risk. Individual investigators and Institutional Biosafety Committees are urged to devise simple and more effective containment procedures and to submit recommended changes in the *NIH Guidelines* to permit the use of these procedures.

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SECTION III. EXPERIMENTS COVERED BY THE *NIH GUIDELINES*

This section describes six categories of experiments involving recombinant or synthetic nucleic acid molecules: (i) those that require Institutional Biosafety Committee (IBC) approval, RAC review, and NIH Director approval before initiation (see Section III-A), (ii) those that require NIH/OBA and Institutional Biosafety Committee approval before initiation (see Section III-B), (iii) those that require Institutional Biosafety Committee and Institutional Review Board approvals and RAC review before research participant enrollment (see Section III-C), (iv) those that require Institutional Biosafety Committee approval before initiation (see Section III-D), (v) those that require Institutional Biosafety Committee notification simultaneous with initiation (see Section III-E), and (vi) those that are exempt from the *NIH Guidelines* (see Section III-F).

Note: *If an experiment falls into Sections III-A, III-B, or III-C and one of the other sections, the rules pertaining to Sections III-A, III-B, or III-C shall be followed.* If an experiment falls into Section III-F and into either Sections III-D or III-E as well, the experiment is considered exempt from the *NIH Guidelines*.

Any change in containment level, which is different from those specified in the *NIH Guidelines*, may not be initiated without the express approval of NIH/OBA (see Section IV-C-1-b-(2) and its subsections, *Minor Actions*).

Section III-A. Experiments that Require Institutional Biosafety Committee Approval, RAC Review, and NIH Director Approval Before Initiation (See Section IV-C-1-b-(1), Major Actions).

Section III-A-1. Major Actions under the *NIH Guidelines*

Experiments considered as *Major Actions* under the *NIH Guidelines* cannot be initiated without submission of relevant information on the proposed experiment to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax), the publication of the proposal in the *Federal Register* for 15 days of comment, review by RAC, and specific approval by NIH. The containment conditions or stipulation requirements for such experiments will be recommended by RAC and set by NIH at the time of approval. Such experiments require Institutional Biosafety Committee approval before initiation. Specific experiments already approved are included in Appendix D, *Major Actions Taken under the NIH Guidelines*, which may be obtained from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

Section III-A-1-a. The deliberate transfer of a drug resistance trait to microorganisms that are not known to acquire the trait naturally (see Section V-B, *Footnotes and References of Sections I-IV*), if such acquisition could compromise the ability to control disease agents in humans, veterinary medicine, or agriculture, will be reviewed by the RAC.

Consideration should be given as to whether the drug resistance trait to be used in the experiment would render that microorganism resistant to the primary drug available to and/or indicated for certain populations, for example children or pregnant women.

At the request of an Institutional Biosafety Committee, NIH/OBA will make a determination regarding whether a specific experiment involving the deliberate transfer of a drug resistance trait falls under Section III-A-1-a and therefore requires RAC review and NIH Director approval. An Institutional Biosafety Committee may also consult with NIH/OBA regarding experiments that do not meet the requirements of Section III-A-1-a but nonetheless raise important public health issues. NIH/OBA will consult, as needed, with one or more experts, which may include the RAC.

Section III-B. Experiments That Require NIH/OBA and Institutional Biosafety Committee Approval Before Initiation

Experiments in this category cannot be initiated without submission of relevant information on the proposed experiment to NIH/OBA. The containment conditions for such experiments will be determined by NIH/OBA in consultation with *ad hoc* experts. Such experiments require Institutional Biosafety Committee approval before initiation (see Section IV-B-2-b-(1), *Institutional Biosafety Committee*).

Section III-B-1. Experiments Involving the Cloning of Toxin Molecules with LD_{50} of Less than 100 Nanograms per Kilogram Body Weight

Deliberate formation of recombinant or synthetic nucleic acid molecules containing genes for the biosynthesis of toxin molecules lethal for vertebrates at an LD_{50} of less than 100 nanograms per kilogram body weight (e.g., microbial toxins such as the botulinum toxins, tetanus toxin, diphtheria toxin, and *Shigella dysenteriae* neurotoxin). Specific approval has been given for the cloning in *Escherichia coli* K-12 of DNA containing genes coding for the biosynthesis of toxic molecules which are lethal to vertebrates at 100 nanograms to 100 micrograms per kilogram body weight. Specific experiments already approved under this section may be obtained from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

Section III-B-2. Experiments that have been Approved (under Section III-A-1-a) as Major Actions under the *NIH Guidelines*

Upon receipt and review of an application from the investigator, NIH/OBA may determine that a proposed experiment is equivalent to an experiment that has previously been approved by the NIH Director as a Major Action, including experiments approved prior to implementation of these changes. An experiment will only be considered equivalent if, as determined by NIH/OBA, there are no substantive differences and pertinent

information has not emerged since submission of the initial III-A-1-a experiment that would change the biosafety and public health considerations for the proposed experiments. If such a determination is made by NIH/OBA, these experiments will not require review and approval under Section III-A.

Section III-C. Experiments that Require Institutional Biosafety Committee and Institutional Review Board Approvals and RAC Review Before Research Participant Enrollment

Section III-C-1. Experiments Involving the Deliberate Transfer of Recombinant or Synthetic Nucleic Acid Molecules, or DNA or RNA Derived from Recombinant or Synthetic Nucleic Acid Molecules, into One or More Human Research Participants

Human gene transfer is the deliberate transfer into human research participants of either:

1. Recombinant nucleic acid molecules, or DNA or RNA derived from recombinant nucleic acid molecules, or
2. Synthetic nucleic acid molecules, or DNA or RNA derived from synthetic nucleic acid molecules, that meet any one of the following criteria:
    a. Contain more than 100 nucleotides; or
    b. Possess biological properties that enable integration into the genome (e.g., *cis* elements involved in integration); or
    c. Have the potential to replicate in a cell; or
    d. Can be translated or transcribed.

No research participant shall be enrolled (see definition of enrollment in Section I-E-7) until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*).

In its evaluation of human gene transfer proposals, the RAC will consider whether a proposed human gene transfer experiment presents characteristics that warrant public RAC review and discussion (See Appendix M-I-B-2). The process of public RAC review and discussion is intended to foster the safe and ethical conduct of human gene transfer experiments. Public review and discussion of a human gene transfer experiment (and access to relevant information) also serves to inform the public about the technical aspects of the proposal, meaning and significance of the research, and any significant safety, social, and ethical implications of the research.

Public RAC review and discussion of a human gene transfer experiment may be: (1) initiated by the NIH Director; or (2) initiated by the NIH OBA Director following a recommendation to NIH OBA by: (a) three or more RAC members; or (b) a Federal agency other than NIH. After a human gene transfer experiment is reviewed by the RAC at a regularly scheduled meeting, NIH OBA will send a letter, unless NIH OBA determines that there are exceptional circumstances, within 10 working days to the NIH Director, the Principal Investigator, the sponsoring institution, and other DHHS components, as appropriate, summarizing the RAC recommendations.

For a clinical trial site that is added after the RAC review process, no research participant shall be enrolled (see definition of enrollment in Section I-E-7) at the clinical trial site until the following documentation has been submitted to NIH OBA: (1) Institutional Biosafety Committee approval (from the clinical trial site); (2) Institutional Review Board approval; (3) Institutional Review Board-approved informed consent document; (4) curriculum vitae of the Principal Investigator(s) (no more than two pages in biographical sketch format); and (5) NIH grant number(s) if applicable.

In order to maintain public access to information regarding human gene transfer protocols (including protocols that are not publicly reviewed by the RAC), NIH OBA will maintain the documentation described in Appendices M-I through M-V. The information provided in response to Appendix M should not contain any confidential commercial information or trade secrets, enabling all aspects of RAC review to be open to the public.

Note: For specific directives concerning the use of retroviral vectors for gene delivery, consult Appendix B-V-1, *Murine Retroviral Vectors.*

Section III-D. Experiments that Require Institutional Biosafety Committee Approval Before Initiation

Prior to the initiation of an experiment that falls into this category, the Principal Investigator must submit a registration document to the Institutional Biosafety Committee which contains the following information: (i) the source(s) of DNA; (ii) the nature of the inserted DNA sequences; (iii) the host(s) and vector(s) to be used; (iv) if an attempt will be made to obtain expression of a foreign gene, and if so, indicate the protein that will be produced; and (v) the containment conditions that will be implemented as specified in the *NIH Guidelines*. For experiments in this category, the registration document shall be dated, signed by the Principal Investigator, and filed with the Institutional Biosafety Committee. The Institutional Biosafety Committee shall review and approve all experiments in this category prior to their initiation. Requests to decrease the level of containment specified for experiments in this category will be considered by NIH (see Section IV-C-1-b-(2)-(c), *Minor Actions*).

Section III-D-1. Experiments Using Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents as Host-Vector Systems (See Section II-A, Risk Assessment)

Section III-D-1-a. Experiments involving the introduction of recombinant or synthetic nucleic acid molecules into Risk Group 2 agents will usually be conducted at Biosafety Level (BL) 2 containment. Experiments with such agents will usually be conducted with whole animals at BL2 or BL2-N (Animals) containment.

Section III-D-1-b. Experiments involving the introduction of recombinant or synthetic nucleic acid molecules into Risk Group 3 agents will usually be conducted at BL3 containment. Experiments with such agents will usually be conducted with whole animals at BL3 or BL3-N containment.

Section III-D-1-c. Experiments involving the introduction of recombinant or synthetic nucleic acid molecules into Risk Group 4 agents shall be conducted at BL4 containment. Experiments with such agents shall be conducted with whole animals at BL4 or BL4-N containment.

Section III-D-1-d. Containment conditions for experiments involving the introduction of recombinant or synthetic nucleic acid molecules into restricted agents shall be set on a case-by-case basis following NIH/OBA review. A U.S. Department of Agriculture - Animal and Plant Health Inspection Service (USDA/APHIS) permit is required for work with plant or animal pathogens (see Section V-G and V-M, *Footnotes and References of Sections I-IV*). Experiments with such agents shall be conducted with whole animals at BL4 or BL4-N containment.

Section III-D-2. Experiments in Which DNA From Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents is Cloned into Nonpathogenic Prokaryotic or Lower Eukaryotic Host-Vector Systems

Section III-D-2-a. Experiments in which DNA from Risk Group 2 or Risk Group 3 agents (see Section II-A, *Risk Assessment)* is transferred into nonpathogenic prokaryotes or lower eukaryotes may be performed under BL2 containment. Experiments in which DNA from Risk Group 4 agents is transferred into nonpathogenic prokaryotes or lower eukaryotes may be performed under BL2 containment after demonstration that only a totally and irreversibly defective fraction of the agent's genome is present in a given recombinant. In the absence of such a demonstration, BL4 containment shall be used. The Institutional Biosafety Committee may approve the specific lowering of containment for particular experiments to BL1. Many experiments in this category are exempt from the *NIH Guidelines* (see Section III-F, *Exempt Experiments*). Experiments involving the formation of recombinant or synthetic nucleic acid molecules for certain genes coding for molecules toxic for vertebrates require NIH/OBA approval (see Section III-B-1, *Experiments Involving the Cloning of Toxin Molecules with LD_{50} of Less than 100 Nanograms Per Kilogram Body Weight*) or shall be conducted under NIH specified conditions as described in Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*.

Section III-D-2-b. Containment conditions for experiments in which DNA from restricted agents is transferred into nonpathogenic prokaryotes or lower eukaryotes shall be determined by NIH/OBA following a case-by-case review (see Section V-L, *Footnotes and References of Sections I-IV*). A U.S. Department of Agriculture permit is required for work with plant or animal pathogens (see Section V-G, *Footnotes and References of Sections I-IV*).

Section III-D-3. Experiments Involving the Use of Infectious DNA or RNA Viruses or Defective DNA or RNA Viruses in the Presence of Helper Virus in Tissue Culture Systems

Caution: Special care should be used in the evaluation of containment levels for experiments which are likely to either enhance the pathogenicity (e.g., insertion of a host oncogene) or to extend the host range (e.g., introduction of novel control elements) of viral vectors under conditions that permit a productive infection. In such cases, serious consideration should be given to increasing physical containment by at least one level.

Note: Recombinant or synthetic nucleic acid molecules or nucleic acid molecules derived therefrom, which contain less than two-thirds of the genome of any eukaryotic virus (all viruses from a single Family (see Section V-J, *Footnotes and References of Sections I-IV*) being considered identical (see Section V-K, *Footnotes and References of Sections I-IV*), are considered defective and may be used in the absence of helper under the conditions specified in Section III-E-1, *Experiments Involving the Formation of Recombinant or Synthetic Molecules Containing No More than Two-Thirds of the Genome of any Eukaryotic Virus*.

Section III-D-3-a. Experiments involving the use of infectious or defective Risk Group 2 viruses (see Appendix B-II, *Risk Group 2 Agents*) in the presence of helper virus may be conducted at BL2.

Section III-D-3-b. Experiments involving the use of infectious or defective Risk Group 3 viruses (see Appendix B-III-D, *Risk Group 3 (RG3) - Viruses and Prions*) in the presence of helper virus may be conducted at BL3.

Section III-D-3-c. Experiments involving the use of infectious or defective Risk Group 4 viruses (see Appendix B-IV-D, *Risk Group 4 (RG4) - Viral Agents*) in the presence of helper virus may be conducted at BL4.

Section III-D-3-d. Experiments involving the use of infectious or defective restricted poxviruses (see Sections V-A and V-L, *Footnotes and References of Sections I-IV*) in the presence of helper virus shall be determined on a case-by-case basis following NIH/OBA review. A U.S. Department of Agriculture permit is required for work with plant or animal pathogens (see Section V-G, *Footnotes and References of Sections I-IV*).

Section III-D-3-e. Experiments involving the use of infectious or defective viruses in the presence of helper virus which are not covered in Sections III-D-3-a through III-D-3-d may be conducted at BL1.

Section III-D-4. Experiments Involving Whole Animals

This section covers experiments involving whole animals in which the animal's genome has been altered by stable introduction of recombinant or synthetic nucleic acid molecules, or nucleic acids derived therefrom, into the germ-line (transgenic animals) and experiments involving viable recombinant or synthetic nucleic acid molecule-modified microorganisms tested on whole animals. For the latter, other than viruses which are only vertically transmitted, the experiments may *not* be conducted at BL1-N containment. A minimum containment of BL2 or BL2-N is required.

Caution - Special care should be used in the evaluation of containment conditions for some experiments with transgenic animals. For example, such experiments might lead to the creation of novel mechanisms or increased transmission of a recombinant pathogen or production of undesirable traits in the host animal. In such cases, serious consideration should be given to increasing the containment conditions.

Section III-D-4-a. Recombinant or synthetic nucleic acid molecules, or DNA or RNA molecules derived therefrom, from any source except for greater than two-thirds of eukaryotic viral genome may be transferred to any non-human vertebrate or any invertebrate organism and propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study (see Section V-B, *Footnotes and References of Sections I-IV*). Animals that contain sequences from viral vectors, which do not lead to transmissible infection either directly or indirectly as a result of complementation or recombination in animals, may be propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study. Experiments involving the introduction of other sequences from eukaryotic viral genomes into animals are covered under Section III-D-4-b, *Experiments Involving Whole Animals*. For experiments involving recombinant or synthetic nucleic acid molecule-modified Risk Groups 2, 3, 4, or restricted organisms, see Sections V-A, V-G, and V-L, *Footnotes and References of Sections I-IV*. It is important that the investigator demonstrate that the fraction of the viral genome being utilized does not lead to productive infection. A U.S. Department of Agriculture permit is required for work with plant or animal

pathogens (see Section V-G, *Footnotes and References of Sections I-IV*).

Section III-D-4-b. For experiments involving recombinant or synthetic nucleic acid molecules, or DNA or RNA derived therefrom, involving whole animals, including transgenic animals, and not covered by Section III-D-1, *Experiments Using Human or Animal Pathogens (Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents as Host-Vector Systems)*, or Section III-D-4-a, the appropriate containment shall be determined by the Institutional Biosafety Committee.

Section III-D-4-c. Exceptions under Section III-D-4, *Experiments Involving Whole Animals*

Section III-D-4-c-(1). Experiments involving the generation of transgenic rodents that require BL1 containment are described under Section III-E-3, *Experiments Involving Transgenic Rodents.*

Section III-D-4-c-(2). The purchase or transfer of transgenic rodents is exempt from the *NIH Guidelines* under Section III-F, *Exempt Experiments* (see Appendix C-VI, *The Purchase or Transfer of Transgenic Rodents*).

Section III-D-5. Experiments Involving Whole Plants

Experiments to genetically engineer plants by recombinant or synthetic nucleic acid molecule methods, to use such plants for other experimental purposes (e.g., response to stress), to propagate such plants, or to use plants together with microorganisms or insects containing recombinant or synthetic nucleic acid molecules, may be conducted under the containment conditions described in Sections III-D-5-a through III-D-5-e. If experiments involving whole plants are not described in Section III-D-5 and do not fall under Sections III-A, III-B, III-D or III-F, they are included in Section III-E.

NOTE - For recombinant or synthetic nucleic acid molecule experiments falling under Sections III-D-5-a through III-D-5-d, physical containment requirements may be reduced to the next lower level by appropriate biological containment practices, such as conducting experiments on a virus with an obligate insect vector in the absence of that vector or using a genetically attenuated strain.

Section III-D-5-a. BL3-P (Plants) or BL2-P + biological containment is recommended for experiments involving most exotic (see Section V-M, *Footnotes and References of Sections I-IV*) infectious agents with recognized potential for serious detrimental impact on managed or natural ecosystems when recombinant or synthetic nucleic acid molecule techniques are associated with whole plants.

Section III-D-5-b. BL3-P or BL2-P + biological containment is recommended for experiments involving plants containing cloned genomes of readily transmissible exotic (see Section V-M, *Footnotes and References of Sections I-IV*) infectious agents with recognized potential for serious detrimental effects on managed or natural ecosystems in which there exists the possibility of reconstituting the complete and functional genome of the infectious agent by genomic complementation *in planta.*

Section III-D-5-c. BL4-P containment is recommended for experiments with a small number of readily transmissible exotic (see Section V-M, *Footnotes and References of Sections I-IV*) infectious agents, such as the soybean rust fungus (*Phakospora pachyrhizi*) and maize streak or other viruses in the presence of their specific arthropod vectors, that have the potential of being serious pathogens of major U.S. crops.

Section III-D-5-d. BL3-P containment is recommended for experiments involving sequences encoding potent vertebrate toxins introduced into plants or associated organisms. Recombinant or synthetic nucleic acid molecules containing genes for the biosynthesis of toxin molecules lethal for vertebrates at an LD_{50} of <100 nanograms per kilogram body weight fall under Section III-B-1, *Experiments Involving the Cloning of Toxin Molecules with LD_{50} of Less than 100 Nanograms Per Kilogram Body Weight*, and require NIH/OBA and Institutional Biosafety Committee approval before initiation.

Section III-D-5-e. BL3-P or BL2-P + biological containment is recommended for experiments with microbial pathogens of insects or small animals associated with plants if the recombinant or synthetic nucleic acid molecule-modified organism has a recognized potential for serious detrimental impact on managed or natural ecosystems.

Section III-D-6. Experiments Involving More than 10 Liters of Culture

The appropriate containment will be decided by the Institutional Biosafety Committee. Where appropriate, Appendix K, *Physical Containment for Large Scale Uses of Organisms Containing Recombinant or Synthetic Recombinant or synthetic nucleic acid Molecules*, shall be used. Appendix K describes containment conditions Good Large Scale Practice through BL3-Large Scale.

Section III-D-7. Experiments Involving Influenza Viruses

Experiments with influenza viruses generated by recombinant or synthetic methods (e.g., generation by reverse genetics of chimeric viruses with reassorted segments, introduction of specific mutations) shall be conducted at the biosafety level containment corresponding to the Risk Group of the virus that was the source of the majority of segments in the recombinant or synthetic virus (e.g., experiments with viruses containing a majority of segments from a RG3 virus shall be conducted at BL3). Experiments with influenza viruses containing genes or segments from 1918-1919 H1N1 (1918 H1N1), human H2N2 (1957-1968) and highly pathogenic avian influenza H5N1 strains within the Goose/Guangdong/96-like H5 lineage (HPAI H5N1) shall be conducted at BL3 enhanced containment (see Appendix G-II-C-5, Biosafety Level 3 Enhanced for Research Involving Risk Group 3 Influenza Viruses) unless indicated below.

Section III-D-7-a. Human H2N2 (1957-1968). Experiments with influenza viruses containing the H2 hemagglutinin (HA) segment shall be conducted at BL3 enhanced (see Appendix G-II-C-5, Biosafety Level 3 Enhanced for Research Involving Risk Group 3 Influenza Viruses). Experiments with the H2 HA gene in cold-adapted, live attenuated vaccine strains (e.g., A/Ann Arbor/6/60 H2N2) may be conducted at BL2 containment provided segments with mutations conferring temperature sensitivity and attenuation are not altered in the recombinant or synthetic virus. Experiments with Risk Group 2 influenza viruses containing genes from human H2N2 other than the HA gene can be worked on at BL2.

Section III-D-7-b. Highly Pathogenic Avian Influenza H5N1 strains within the Goose/Guangdong/96-like H5 lineage (HPAI H5N1). Experiments involving influenza viruses containing a majority of genes and/or segments from a HPAI H5N1 influenza virus shall be conducted at BL3 enhanced containment, (see Appendix G-II-C-5, Biosafety Level 3 Enhanced for Research Involving Risk Group 3 Influenza Viruses). Experiments involving influenza viruses containing a minority of genes and/or segments from a HPAI H5N1 influenza virus shall be conducted at BL3 enhanced unless a risk assessment performed by the IBC determines that they can be conducted safely at biosafety level 2 and after they have been excluded pursuant to 9 CFR 121.3(e). OBA is available to IBCs to provide consultation with the RAC and influenza virus experts when risk assessments are being made to determine the appropriate biocontainment for experiments with influenza viruses containing a minority of gene/segments from HPAI H5N1. Such experiments may be performed at BL3 enhanced containment or containment may be lowered to biosafety level 2, the level of containment for most research with other influenza viruses. (USDA/APHIS regulations and decisions on lowering containment also apply.) In deciding to lower containment, the IBC should consider whether, in at least two animal models (e.g., ferret, mouse, Syrian golden hamster, cotton rat, non-human primates), there is evidence that the resulting influenza virus shows reduced replication and virulence compared to the parental RG3 virus at relevant doses. This should be determined by measuring biological indices appropriate for the specific animal model (e.g., severe weight loss, elevated temperature, mortality or neurological symptoms).

Section III-D-7-c. 1918 H1N1. Experiments involving influenza viruses containing any gene or segment from 1918 H1N1 shall be conducted at BL3 enhanced containment (see Appendix G-II-C-5, Biosafety Level 3 Enhanced for Research Involving Risk Group 3 Influenza Viruses).

Section III-D-7-d. Antiviral Susceptibility and Containment. The availability of antiviral drugs as preventive and therapeutic measures is an important safeguard for experiments with 1918 H1N1, HPAI H5N1, and human H2N2 (1957-1968). If an influenza virus containing genes from one of these viruses is resistant to both classes of current antiviral agents, adamantanes and neuraminidase inhibitors, higher containment may be required based on the risk assessment considering transmissibility to humans, virulence, pandemic potential, alternative antiviral agents if available, etc.

Experiments with 1918 H1N1, human H2N2 (1957-1968) or HPAI H5N1 that are designed to create resistance to neuraminidase inhibitors or other effective antiviral agents (including investigational antiviral agents being developed for influenza) would be subject to Section III-A-1 (Major Actions) and require RAC review and NIH

Director approval. As per Section I-A-1 of the *NIH Guidelines*, if the agent is a Select Agent, the NIH will defer to the appropriate Federal agency (HHS or USDA Select Agent Divisions) on such experiments.

Section III-E. Experiments that Require Institutional Biosafety Committee Notice Simultaneous with Initiation

Experiments not included in Sections III-A, III-B, III-C, III-D, III-F, and their subsections are considered in Section III-E. All such experiments may be conducted at BL1 containment. For experiments in this category, a registration document (see Section III-D, *Experiments that Require Institutional Biosafety Committee Approval Before Initiation*) shall be dated and signed by the investigator and filed with the local Institutional Biosafety Committee at the time the experiment is initiated. The Institutional Biosafety Committee reviews and approves all such proposals, but Institutional Biosafety Committee review and approval prior to initiation of the experiment is not required (see Section IV-A, *Policy*). For example, experiments in which all components derived from non-pathogenic prokaryotes and non-pathogenic lower eukaryotes fall under Section III-E and may be conducted at BL1 containment.

Section III-E-1. Experiments Involving the Formation of Recombinant or Synthetic Nucleic Acid Molecules Containing No More than Two-Thirds of the Genome of any Eukaryotic Virus

Recombinant or synthetic nucleic acid molecules containing no more than two-thirds of the genome of any eukaryotic virus (all viruses from a single Family being considered identical [see Section V-J, *Footnotes and References of Sections I-IV*]) may be propagated and maintained in cells in tissue culture using BL1 containment. For such experiments, it must be demonstrated that the cells lack helper virus for the specific Families of defective viruses being used. If helper virus is present, procedures specified under Section III-D-3, *Experiments Involving the Use of Infectious Animal or Plant DNA or RNA Viruses or Defective Animal or Plant DNA or RNA Viruses in the Presence of Helper Virus in Tissue Culture Systems*, should be used. The DNA may contain fragments of the genome of viruses from more than one Family but each fragment shall be less than two-thirds of a genome.

Section III-E-2. Experiments Involving Whole Plants

This section covers experiments involving nucleic acid molecule-modified whole plants, and/or experiments involving recombinant or synthetic nucleic acid molecule-modified organisms associated with whole plants, except those that fall under Section III-A, III-B, III-D, or III-F. It should be emphasized that knowledge of the organisms and judgment based on accepted scientific practices should be used in all cases in selecting the appropriate level of containment. For example, if the genetic modification has the objective of increasing pathogenicity or converting a non-pathogenic organism into a pathogen, then a higher level of containment may be appropriate depending on the organism, its mode of dissemination, and its target organisms. By contrast, a lower level of containment may be appropriate for small animals associated with many types of recombinant or synthetic nucleic acid molecule-modified plants.

Section III-E-2-a. BL1-P is recommended for all experiments with recombinant or synthetic recombinant or synthetic nucleic acid molecule-containing plants and plant-associated microorganisms not covered in Section III-E-2-b or other sections of the *NIH Guidelines*. Examples of such experiments are those involving recombinant or synthetic nucleic acid molecule-modified plants that are not noxious weeds or that cannot interbreed with noxious weeds in the immediate geographic area, and experiments involving whole plants and recombinant or synthetic nucleic acid molecule-modified non-exotic (see Section V-M, *Footnotes and References of Sections I-IV*) microorganisms that have no recognized potential for rapid and widespread dissemination or for serious detrimental impact on managed or natural ecosystems (e.g., *Rhizobium* spp. and *Agrobacterium* spp.).

Section III-E-2-b. BL2-P or BL1-P + biological containment is recommended for the following experiments:

Section III-E-2-b-(1). Plants modified by recombinant or synthetic nucleic acid molecules that are noxious weeds or can interbreed with noxious weeds in the immediate geographic area.

Section III-E-2-b-(2). Plants in which the introduced DNA represents the complete genome of a non-exotic infectious agent (see Section V-M, *Footnotes and References of Sections I-IV*).

Section III-E-2-b-(3). Plants associated with recombinant or synthetic nucleic acid molecule-modified non-exotic microorganisms that have a recognized potential for serious detrimental impact on managed or natural ecosystems (see Section V-M, *Footnotes and References of Sections I-IV*).

Section III-E-2-b-(4). Plants associated with recombinant or synthetic nucleic acid molecule-modified exotic microorganisms that have no recognized potential for serious detrimental impact on managed or natural ecosystems (see Section V-M, *Footnotes and References of Sections I-IV*).

Section III-E-2-b-(5). Experiments with recombinant or synthetic nucleic acid molecule-modified arthropods or small animals associated with plants, or with arthropods or small animals with recombinant or synthetic nucleic acid molecule-modified microorganisms associated with them if the recombinant or synthetic nucleic acid molecule-modified microorganisms have no recognized potential for serious detrimental impact on managed or natural ecosystems (see Section V-M, *Footnotes and References of Sections I-IV*).

Section III-E-3. Experiments Involving Transgenic Rodents

This section covers experiments involving the generation of rodents in which the animal's genome has been altered by stable introduction of recombinant or synthetic nucleic acid molecules, or nucleic acids derived therefrom, into the germ-line (transgenic rodents). Only experiments that require BL1 containment are covered under this section; experiments that require BL2, BL3, or BL4 containment are covered under Section III-D-4, *Experiments Involving Whole Animals*.

Section III-E-3-a. Experiments involving the breeding of certain BL1 transgenic rodents are exempt under Section III-F, *Exempt Experiments* (See Appendix C-VII, *Generation of BL1 Transgenic Rodents via Breeding*).

Section III-F. Exempt Experiments

The following recombinant or synthetic nucleic acid molecules are exempt from the *NIH Guidelines* and registration with the Institutional Biosafety Committee is not required; however, other federal and state standards of biosafety may still apply to such research (for example, the Centers for Disease Control and Prevention (CDC)/NIH publication Biosafety in Microbiological and Biomedical Laboratories).

Section III-F-1. Those synthetic nucleic acids that: (1) can neither replicate nor generate nucleic acids that can replicate in any living cell (e.g., oligonucleotides or other synthetic nucleic acids that do not contain an origin of replication or contain elements known to interact with either DNA or RNA polymerase), and (2) are not designed to integrate into DNA, and (3) do not produce a toxin that is lethal for vertebrates at an LD50 of less than 100 nanograms per kilogram body weight. If a synthetic nucleic acid is deliberately transferred into one or more human research participants and meets the criteria of Section III-C, it is not exempt under this Section.

Section III-F-2. Those that are not in organisms, cells, or viruses and that have not been modified or manipulated (e.g., encapsulated into synthetic or natural vehicles) to render them capable of penetrating cellular membranes.

Section III-F-3. Those that consist solely of the exact recombinant or synthetic nucleic acid sequence from a single source that exists contemporaneously in nature.

Section III-F-4. Those that consist entirely of nucleic acids from a prokaryotic host, including its indigenous plasmids or viruses when propagated only in that host (or a closely related strain of the same species), or when transferred to another host by well established physiological means.

Section III-F-5. Those that consist entirely of nucleic acids from a eukaryotic host including its chloroplasts, mitochondria, or plasmids (but excluding viruses) when propagated only in that host (or a closely related strain of the same species).

Section III-F-6. Those that consist entirely of DNA segments from different species that exchange DNA by known physiological processes, though one or more of the segments may be a synthetic equivalent. A list of such exchangers will be prepared and periodically revised by the NIH Director with advice of the RAC after appropriate notice and opportunity for public comment (see Section IV-C-1-b-(1)-(c), *Major Actions*). See Appendices A-I through A-VI, *Exemptions under Section III-F-6--Sublists of Natural Exchangers*, for a list of

natural exchangers that are exempt from the *NIH Guidelines*.

Section III-F-7. Those genomic DNA molecules that have acquired a transposable element, provided the transposable element does not contain any recombinant and/or synthetic DNA.

Section III-F-8. Those that do not present a significant risk to health or the environment (see Section IV-C-1-b-(1)-(c), *Major Actions*), as determined by the NIH Director, with the advice of the RAC, and following appropriate notice and opportunity for public comment. See Appendix C, *Exemptions under Section III-F-8* for other classes of experiments which are exempt from the *NIH Guidelines*.

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SECTION IV. ROLES AND RESPONSIBILITIES

Section IV-A. Policy

The safe conduct of experiments involving recombinant or synthetic nucleic acid molecules depends on the individual conducting such activities. The *NIH Guidelines* cannot anticipate every possible situation. Motivation and good judgment are the key essentials to protection of health and the environment. The *NIH Guidelines* are intended to assist the institution, Institutional Biosafety Committee, Biological Safety Officer, and the Principal Investigator in determining safeguards that should be implemented. The *NIH Guidelines* will never be complete or final since all conceivable experiments involving recombinant or synthetic nucleic acid molecules cannot be foreseen. The utilization of new genetic manipulation techniques may enable work previously conducted using recombinant means to be accomplished faster, more efficiently, or at larger scale. These techniques have not yet yielded organisms that present safety concerns that fall outside the current risk assessment framework used for recombinant nucleic acid research. Nonetheless, an appropriate risk assessment of experiments involving these techniques must be conducted taking into account the way these approaches may alter the risk assessment. As new techniques develop, the *NIH Guidelines* should be periodically reviewed to determine whether and how such research should be explicitly addressed.

It is the responsibility of the institution and those associated with it to adhere to the intent of the *NIH Guidelines* as well as to their specifics. Therefore, each institution (and the Institutional Biosafety Committee acting on its behalf) is responsible for ensuring that all research with recombinant or synthetic nucleic acid molecules conducted at or sponsored by that institution is conducted in compliance with the *NIH Guidelines*. The following roles and responsibilities constitute an administrative framework in which safety is an essential and integral part of research involving recombinant or synthetic nucleic acid molecules. Further clarifications and interpretations of roles and responsibilities will be issued by NIH as necessary.

Section IV-B. Responsibilities of the Institution

Section IV-B-1. General Information

Each institution conducting or sponsoring recombinant or synthetic nucleic acid molecule research which is covered by the *NIH Guidelines* is responsible for ensuring that the research is conducted in full conformity with the provisions of the *NIH Guidelines*. In order to fulfill this responsibility, the institution shall:

Section IV-B-1-a. Establish and implement policies that provide for the safe conduct of recombinant or synthetic nucleic acid molecule research and that ensure compliance with the *NIH Guidelines*. As part of its general responsibilities for implementing the *NIH Guidelines*, the institution may establish additional procedures, as deemed necessary, to govern the institution and its components in the discharge of its responsibilities under the *NIH Guidelines*. Such procedures may include: (i) statements formulated by the institution for the general implementation of the *NIH Guidelines*, and (ii) any additional precautionary steps the institution deems appropriate.

Section IV-B-1-b. Establish an Institutional Biosafety Committee that meets the requirements set forth in Section IV-B-2-a and carries out the functions detailed in Section IV-B-2-b.

Section IV-B-1-c. Appoint a Biological Safety Officer (who is also a member of the Institutional Biosafety Committee) if the institution: (i) conducts recombinant or synthetic nucleic acid molecule research at Biosafety

Level (BL) 3 or BL4, or (ii) engages in large-scale (greater than 10 liters) research. The Biological Safety Officer carries out the duties specified in Section IV-B-3.

Section IV-B-1-d. Appoint at least one individual with expertise in plant, plant pathogen, or plant pest containment principles (who is a member of the Institutional Biosafety Committee) if the institution conducts recombinant or synthetic nucleic acid molecule research that requires Institutional Biosafety Committee approval in accordance with Appendix P, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Plants.*

Section IV-B-1-e. Appoint at least one individual with expertise in animal containment principles (who is a member of the Institutional Biosafety Committee) if the institution conducts recombinant or synthetic nucleic acid molecule research that requires Institutional Biosafety Committee approval in accordance with Appendix Q, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Animals.*

Section IV-B-1-f. Ensure that when the institution participates in or sponsors recombinant or synthetic nucleic acid molecule research involving human subjects: (i) the Institutional Biosafety Committee has adequate expertise and training (using *ad hoc* consultants as deemed necessary), (ii) all aspects of Appendix M have been appropriately addressed by the Principal Investigator; and (iii) no research participant shall be enrolled (see definition of enrollment in Section I-E-7) in a human gene transfer experiment until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*), Institutional Biosafety Committee approval has been obtained,

Institutional Review Board approval has been obtained, and all applicable regulatory authorizations have been obtained. Institutional Biosafety Committee approval must be obtained from each institution at which recombinant or synthetic nucleic acids will be administered to human subjects (as opposed to each institution involved in the production of vectors for human application and each institution at which there is *ex vivo* transduction of recombinant or synthetic nucleic acid molecule material into target cells for human application).

Section IV-B-1-g. Assist and ensure compliance with the *NIH Guidelines* by Principal Investigators conducting research at the institution as specified in Section IV-B-7.

Section IV-B-1-h. Ensure appropriate training for the Institutional Biosafety Committee Chair and members, Biological Safety Officer and other containment experts (when applicable), Principal Investigators, and laboratory staff regarding laboratory safety and implementation of the *NIH Guidelines*. The Institutional Biosafety Committee Chair is responsible for ensuring that Institutional Biosafety Committee members are appropriately trained. The Principal Investigator is responsible for ensuring that laboratory staff are appropriately trained. The institution is responsible for ensuring that the Principal Investigator has sufficient training; however, this responsibility may be delegated to the Institutional Biosafety Committee.

Section IV-B-1-i. Determine the necessity for health surveillance of personnel involved in connection with individual recombinant or synthetic nucleic acid molecule projects; and if appropriate, conduct a health surveillance program for such projects. The institution shall establish and maintain a health surveillance program for personnel engaged in large-scale research or production activities involving viable organisms containing recombinant or synthetic nucleic acid molecules which require BL3 containment at the laboratory scale. The institution shall establish and maintain a health surveillance program for personnel engaged in animal research involving viable recombinant or synthetic nucleic acid molecule-containing microorganisms that require BL3 or greater containment in the laboratory. The *Laboratory Safety Monograph* discusses various components of such a program (e.g., records of agents handled, active investigation of relevant illnesses, and the maintenance of serial serum samples for monitoring serologic changes that may result from the employees' work experience). Certain medical conditions may place a laboratory worker at increased risk in any endeavor where infectious agents are handled. Examples cited in the *Laboratory Safety Monograph* include gastrointestinal disorders and treatment with steroids, immunosuppressive drugs, or antibiotics. Workers with such disorders or treatment should be evaluated to determine whether they should be engaged in research with potentially hazardous organisms during their treatment or illness. Copies of the *Laboratory Safety Monograph* are available from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

Section IV-B-1-j. Report any significant problems, violations of the *NIH Guidelines*, or any significant research-related accidents and illnesses to NIH/OBA within thirty days, unless the institution determines that a report has already been filed by the Principal Investigator or Institutional Biosafety Committee. Reports shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

Section IV-B-2. Institutional Biosafety Committee (IBC)

The institution shall establish an Institutional Biosafety Committee whose responsibilities need not be restricted to recombinant or synthetic nucleic acid molecule research. The Institutional Biosafety Committee shall meet the following requirements:

Section IV-B-2-a. Membership and Procedures

Section IV-B-2-a-(1). The Institutional Biosafety Committee must be comprised of no fewer than five members so selected that they collectively have experience and expertise in recombinant or synthetic nucleic acid molecule technology and the capability to assess the safety of recombinant or synthetic nucleic acid molecule research and to identify any potential risk to public health or the environment. At least two members shall not be affiliated with the institution (apart from their membership on the Institutional Biosafety Committee) and who represent the interest of the surrounding community with respect to health and protection of the environment (e.g., officials of state or local public health or environmental protection agencies, members of other local governmental bodies, or persons active in medical, occupational health, or environmental concerns in the community). The Institutional Biosafety Committee shall include at least one individual with expertise in plant, plant pathogen, or plant pest containment principles when experiments utilizing Appendix P, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research*

Involving Plants, require prior approval by the Institutional Biosafety Committee. The Institutional Biosafety Committee shall include at least one scientist with expertise in animal containment principles when experiments utilizing Appendix Q, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Animals*, require Institutional Biosafety Committee prior approval. When the institution conducts recombinant or synthetic nucleic acid molecule research at BL3, BL4, or Large Scale (greater than 10 liters), a Biological Safety Officer is mandatory and shall be a member of the Institutional Biosafety Committee (see Section IV-B-3, *Biological Safety Officer*). When the institution participates in or sponsors recombinant or synthetic nucleic acid molecule research involving human research participants, the institution must ensure that: (i) the Institutional Biosafety Committee has adequate expertise and training (using *ad hoc* consultants as deemed necessary); (ii) all aspects of Appendix M have been appropriately addressed by the Principal Investigator; (iii) no research participant shall be enrolled (see definition of enrollment in Section I-E-7) in a human gene transfer experiment until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*); and (iv) final IBC approval is granted only after the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*). Institutional Biosafety Committee approval must be obtained from the institution at which recombinant or synthetic nucleic acid molecule material will be administered to human research participants (rather than the site involved in manufacturing gene transfer products).

Note: Individuals, corporations, and institutions not otherwise covered by the *NIH Guidelines*, are encouraged to adhere to the standards and procedures set forth in Sections I through IV (see Section IV-D, *Voluntary Compliance*. The policy and procedures for establishing an Institutional Biosafety Committee under *Voluntary Compliance*, are specified in Section IV-D-2, *Institutional Biosafety Committee Approval*).

Section IV-B-2-a-(2). In order to ensure the competence necessary to review and approve recombinant or synthetic nucleic acid molecule activities, it is recommended that the Institutional Biosafety Committee: (i) include persons with expertise in recombinant or synthetic nucleic acid molecule technology, biological safety, and physical containment; (ii) include or have available as consultants persons knowledgeable in institutional commitments and policies, applicable law, standards of professional conduct and practice, community attitudes, and the environment, and (iii) include at least one member representing the laboratory technical staff.

Section IV-B-2-a-(3). The institution shall file an annual report with NIH/OBA which includes: (i) a roster of all Institutional Biosafety Committee members clearly indicating the Chair, contact person, Biological Safety Officer (if applicable), plant expert (if applicable), animal expert (if applicable), human gene therapy expertise or *ad hoc*

consultant (if applicable); and (ii) biographical sketches of all Institutional Biosafety Committee members (including community members).

Section IV-B-2-a-(4). No member of an Institutional Biosafety Committee may be involved (except to provide information requested by the Institutional Biosafety Committee) in the review or approval of a project in which he/she has been or expects to be engaged or has a direct financial interest.

Section IV-B-2-a-(5). The institution, that is ultimately responsible for the effectiveness of the Institutional Biosafety Committee, may establish procedures that the Institutional Biosafety Committee shall follow in its initial and continuing review and approval of applications, proposals, and activities.

Section IV-B-2-a-(6). When possible and consistent with protection of privacy and proprietary interests, the institution is encouraged to open its Institutional Biosafety Committee meetings to the public.

Section IV-B-2-a-(7). Upon request, the institution shall make available to the public all Institutional Biosafety Committee meeting minutes and any documents submitted to or received from funding agencies which the latter are required to make available to the public. If public comments are made on Institutional Biosafety Committee actions, the institution shall forward both the public comments and the Institutional Biosafety Committee's response to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

Section IV-B-2-b. Functions

On behalf of the institution, the Institutional Biosafety Committee is responsible for:

Section IV-B-2-b-(1). Reviewing recombinant or synthetic nucleic acid molecule research conducted at or sponsored by the institution for compliance with the *NIH Guidelines* as specified in Section III, *Experiments Covered by the NIH Guidelines*, and approving those research projects that are found to conform with the *NIH Guidelines*. This review shall include: (i) independent assessment of the containment levels required by the *NIH Guidelines* for the proposed research; (ii) assessment of the facilities, procedures, practices, and training and expertise of personnel involved in recombinant or synthetic nucleic acid molecule research; (iii) ensuring that all aspects of Appendix M have been appropriately addressed by the Principal Investigator; (iv) ensuring that no research participant is enrolled (see definition of enrollment in Section I-E-7) in a human gene transfer experiment until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*), Institutional Biosafety Committee approval (from the clinical trial site) has been obtained, Institutional Review Board approval has been obtained, and all applicable regulatory authorizations have been obtained; (v) for human gene transfer protocols selected for public RAC review and discussion, consideration of the issues raised and recommendations made as a result of this review and consideration of the Principal Investigator's response to the RAC recommendations; (vi) ensuring that final IBC approval is granted only after the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*); and (vii) ensuring compliance with all surveillance, data reporting, and adverse event reporting requirements set forth in the *NIH Guidelines*.

Section IV-B-2-b-(2). Notifying the Principal Investigator of the results of the Institutional Biosafety Committee's review and approval.

Section IV-B-2-b-(3). Lowering containment levels for certain experiments as specified in Section III-D-2-a, *Experiments in which DNA from Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents is Cloned into Nonpathogenic Prokaryotic or Lower Eukaryotic Host-Vector Systems.*

Section IV-B-2-b-(4). Setting containment levels as specified in Sections III-D-4-b, *Experiments Involving Whole Animals*, and III-D-5, *Experiments Involving Whole Plants.*

Section IV-B-2-b-(5). Periodically reviewing recombinant or synthetic nucleic acid molecule research conducted at the institution to ensure compliance with the *NIH Guidelines*.

Section IV-B-2-b-(6). Adopting emergency plans covering accidental spills and personnel contamination resulting from recombinant or synthetic nucleic acid molecule research.

Note: The *Laboratory Safety Monograph* describes basic elements for developing specific procedures dealing with major spills of potentially hazardous materials in the laboratory, including information and references about decontamination and emergency plans. The NIH and the CDC are available to provide consultation and direct assistance, if necessary, as posted in the *Laboratory Safety Monograph*. The institution shall cooperate with the state and local public health departments by reporting any significant research-related illness or accident that may be hazardous to the public health.

Section IV-B-2-b-(7). Reporting any significant problems with or violations of the *NIH Guidelines* and any significant research-related accidents or illnesses to the appropriate institutional official and NIH/OBA within 30 days, unless the Institutional Biosafety Committee determines that a report has already been filed by the Principal Investigator. Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

Section IV-B-2-b-(8). The Institutional Biosafety Committee may not authorize initiation of experiments which are not explicitly covered by the *NIH Guidelines* until NIH (with the advice of the RAC when required) establishes the containment requirement.

Section IV-B-2-b-(9). Performing such other functions as may be delegated to the Institutional Biosafety Committee under Section IV-B-2, *Institutional Biosafety Committee*.

Section IV-B-3. Biological Safety Officer (BSO)

Section IV-B-3-a. The institution shall appoint a Biological Safety Officer if it engages in large-scale research or production activities involving viable organisms containing recombinant or synthetic nucleic acid molecules.

Section IV-B-3-b. The institution shall appoint a Biological Safety Officer if it engages in recombinant or synthetic nucleic acid molecule research at BL3 or BL4. The Biological Safety Officer shall be a member of the Institutional Biosafety Committee.

Section IV-B-3-c. The Biological Safety Officer's duties include, but are not be limited to:

Section IV-B-3-c-(1). Periodic inspections to ensure that laboratory standards are rigorously followed;

Section IV-B-3-c-(2). Reporting to the Institutional Biosafety Committee and the institution any significant problems, violations of the *NIH Guidelines*, and any significant research-related accidents or illnesses of which the Biological Safety Officer becomes aware unless the Biological Safety Officer determines that a report has already been filed by the Principal Investigator;

Section IV-B-3-c-(3). Developing emergency plans for handling accidental spills and personnel contamination and investigating laboratory accidents involving recombinant or synthetic nucleic acid molecule research;

Section IV-B-3-c-(4). Providing advice on laboratory security;

Section IV-B-3-c-(5). Providing technical advice to Principal Investigators and the Institutional Biosafety Committee on research safety procedures.

Note: See the *Laboratory Safety Monograph* for additional information on the duties of the Biological Safety Officer.

Section IV-B-4. Plant, Plant Pathogen, or Plant Pest Containment Expert

When the institution conducts recombinant or synthetic nucleic acid molecule research that requires Institutional Biosafety Committee approval in accordance with Appendix P, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Plants*, the institution shall appoint at least one individual with expertise in plant, plant pathogen, or plant pest containment principles (who is a member of the Institutional Biosafety Committee).

Section IV-B-5. Animal Containment Expert

When the institution conducts recombinant or synthetic nucleic acid molecule research that requires Institutional Biosafety Committee approval in accordance with Appendix Q, *Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Animals*, the institution shall appoint at least one individual with expertise in animal containment principles (who is a member of the Institutional Biosafety Committee).

Section IV-B-6. Human Gene Therapy Expertise

When the institution participates in or sponsors recombinant or synthetic nucleic acid molecule research involving human subjects, the institution must ensure that: (i) the Institutional Biosafety Committee has adequate expertise and training (using *ad hoc* consultants as deemed necessary) and (ii) all aspects of Appendix M, *Points to Consider in the Design and Submission of Protocols for the Transfer of Recombinant or Synthetic Nucleic Acid Molecules into One or More Human Subjects (Points to Consider)* , have been appropriately addressed by the Principal Investigator prior to submission to NIH/OBA.

Section IV-B-7. Principal Investigator (PI)

On behalf of the institution, the Principal Investigator is responsible for full compliance with the *NIH Guidelines* in the conduct of recombinant or synthetic nucleic acid molecule research. A Principal Investigator engaged in human gene transfer research may delegate to another party, such as a corporate sponsor, the reporting functions set forth in Appendix M, with written notification to the NIH OBA of the delegation and of the name(s), address, telephone, and fax numbers of the contact. The Principal Investigator is responsible for ensuring that the reporting requirements are fulfilled and will be held accountable for any reporting lapses.

Section IV-B-7-a. General Responsibilities

As part of this general responsibility, the Principal Investigator shall:

Section IV-B-7-a-(1). Initiate or modify no recombinant or synthetic nucleic acid molecule research which requires Institutional Biosafety Committee approval prior to initiation (see Sections III-A, III-B, III-C, III-D, and III-E, *Experiments Covered by the NIH Guidelines*) until that research or the proposed modification thereof has been approved by the Institutional Biosafety Committee and has met all other requirements of the *NIH Guidelines*;

Section IV-B-7-a-(2). Determine whether experiments are covered by Section III-E, *Experiments that Require Institutional Biosafety Committee Notice Simultaneous with Initiation*, and ensure that the appropriate procedures are followed;

Section IV-B-7-a-(3). Report any significant problems, violations of the *NIH Guidelines*, or any significant research-related accidents and illnesses to the Biological Safety Officer (where applicable), Greenhouse/Animal Facility Director (where applicable), Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable) within 30 days. Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax);

Section IV-B-7-a-(4). Report any new information bearing on the *NIH Guidelines* to the Institutional Biosafety Committee and to NIH/OBA (reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax);

Section IV-B-7-a-(5). Be adequately trained in good microbiological techniques;

Section IV-B-7-a-(6). Adhere to Institutional Biosafety Committee approved emergency plans for handling accidental spills and personnel contamination; and

Section IV-B-7-a-(7). Comply with shipping requirements for recombinant or synthetic nucleic acid molecules (see Appendix H, *Shipment*, for shipping requirements and the *Laboratory Safety Monograph* for technical recommendations).

Section IV-B-7-b. Information to Be Submitted by the Principal Investigator to NIH OBA

The Principal Investigator shall:

Section IV-B-7-b-(1). Submit information to NIH/OBA for certification of new host-vector systems;

Section IV-B-7-b-(2). Petition NIH/OBA, with notice to the Institutional Biosafety Committee, for proposed exemptions to the *NIH Guidelines*;

Section IV-B-7-b-(3). Petition NIH/OBA, with concurrence of the Institutional Biosafety Committee, for approval to conduct experiments specified in Sections III-A-1, *Major Actions Under the NIH Guidelines*, and III-B, *Experiments that Require NIH/OBA and Institutional Biosafety Committee Approval Before Initiation*;

Section IV-B-7-b-(4). Petition NIH/OBA for determination of containment for experiments requiring case-by-case review; and

Section IV-B-7-b-(5). Petition NIH/OBA for determination of containment for experiments not covered by the *NIH Guidelines*.

Section IV-B-7-b-(6). Ensure that all aspects of Appendix M have been appropriately addressed prior to submission of a human gene transfer experiment to NIH OBA, and provide a letter signed by the Principal Investigator(s) on institutional letterhead acknowledging that the documentation being submitted to NIH OBA complies with the requirements set forth in Appendix M. No research participant shall be enrolled (see definition of enrollment in Section I-E-7) in a human gene transfer experiment until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*); IBC approval (from the clinical trial site) has been obtained; Institutional Review Board (IRB) approval has been obtained; and all applicable regulatory authorization(s) have been obtained.

For a clinical trial site that is added after the RAC review process, no research participant shall be enrolled (see definition of enrollment in Section I-E-7) at the clinical trial site until the following documentation has been submitted to NIH OBA: (1) IBC approval (from the clinical trial site); (2) IRB approval; (3) IRB-approved informed consent document; (4) curriculum vitae of the Principal Investigator(s) (no more than two pages in biographical sketch format); and (5) NIH grant number(s) if applicable.

Section IV-B-7-c. Submissions by the Principal Investigator to the Institutional Biosafety Committee

The Principal Investigator shall:

Section IV-B-7-c-(1). Make an initial determination of the required levels of physical and biological containment in accordance with the *NIH Guidelines*;

Section IV-B-7-c-(2). Select appropriate microbiological practices and laboratory techniques to be used for the research;

Section IV-B-7-c-(3). Submit the initial research protocol and any subsequent changes (e.g., changes in the source of DNA or host-vector system), if covered under Sections III-A, III-B, III-C, III-D, or III-E (*Experiments Covered by the NIH Guidelines*), to the Institutional Biosafety Committee for review and approval or disapproval; and

Section IV-B-7-c-(4). Remain in communication with the Institutional Biosafety Committee throughout the conduct of the project.

Section IV-B-7-d. Responsibilities of the Principal Investigator Prior to Initiating Research

The Principal Investigator shall:

Section IV-B-7-d-(1). Make available to all laboratory staff the protocols that describe the potential biohazards and the precautions to be taken;

Section IV-B-7-d-(2). Instruct and train laboratory staff in: (i) the practices and techniques required to ensure safety, and (ii) the procedures for dealing with accidents; and

Section IV-B-7-d-(3). Inform the laboratory staff of the reasons and provisions for any precautionary medical practices advised or requested (e.g., vaccinations or serum collection).

Section IV-B-7-e. Responsibilities of the Principal Investigator During the Conduct of the Research

The Principal Investigator shall:

Section IV-B-7-e-(1). Supervise the safety performance of the laboratory staff to ensure that the required safety practices and techniques are employed;

Section IV-B-7-e-(2). Investigate and report any significant problems pertaining to the operation and implementation of containment practices and procedures in writing to the Biological Safety Officer (where applicable), Greenhouse/Animal Facility Director (where applicable), Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable) (reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax);

Section IV-B-7-e-(3). Correct work errors and conditions that may result in the release of recombinant or synthetic nucleic acid molecule materials; and

Section IV-B-7-e-(4). Ensure the integrity of the physical containment (e.g., biological safety cabinets) and the biological containment (e.g., purity and genotypic and phenotypic characteristics).

Section IV-B-7-e-(5). Comply with reporting requirements for human gene transfer experiments conducted in compliance with the *NIH Guidelines* (see Appendix M-I-C, *Reporting Requirements*).

Section IV-C. Responsibilities of the National Institutes of Health (NIH)

Section IV-C-1. NIH Director

The NIH Director is responsible for: (i) establishing the *NIH Guidelines*, (ii) overseeing their implementation, and (iii) their final interpretation. The NIH Director has responsibilities under the *NIH Guidelines* that involve OBA and RAC. OBA's responsibilities under the *NIH Guidelines* are administrative. Advice from RAC is primarily scientific, technical, and ethical. In certain circumstances, there is specific opportunity for public comment with published response prior to final action.

Section IV-C-1-a. General Responsibilities

The NIH Director is responsible for:

Section IV-C-1-a-(1). Promulgating requirements as necessary to implement the *NIH Guidelines*;

Section IV-C-1-a-(2). Establishing and maintaining RAC to carry out the responsibilities set forth in Section IV-C-2, *Recombinant DNA Advisory Committee* (RAC membership is specified in its charter and in Section IV-C-2);

Section IV-C-1-a-(3). Establishing and maintaining NIH/OBA to carry out the responsibilities defined in Section IV-C-3, *Office of Biotechnology Activities*;

Section IV-C-1-a-(4). Conducting and supporting training programs in laboratory safety for Institutional Biosafety Committee members, Biological Safety Officers and other institutional experts (if applicable), Principal Investigators, and laboratory staff.

Section IV-C-1-a-(5). Establishing and convening Gene Therapy Policy Conferences as described in Appendix L, *Gene Therapy Policy Conferences.*

Section IV-C-1-b. Specific Responsibilities

In carrying out the responsibilities set forth in this section, the NIH Director, or a designee shall weigh each proposed action through appropriate analysis and consultation to determine whether it complies with the *NIH Guidelines* and presents no significant risk to health or the environment.

Section IV-C-1-b-(1). Major Actions

To execute *Major Actions*, the NIH Director shall seek the advice of RAC and provide an opportunity for public and Federal agency comment. Specifically, the Notice of Meeting and *Proposed Actions* shall be published in the *Federal Register* at least 15 days before the RAC meeting. The NIH Director's decision/recommendation (at his/her discretion) may be published in the *Federal Register* for 15 days of comment before final action is taken. The NIH Director's final decision/recommendation, along with responses to public comments, shall be published in the *Federal Register*. The RAC and Institutional Biosafety Committee Chairs shall be notified of the following decisions:

Section IV-C-1-b-(1)-(a). Changing containment levels for types of experiments that are specified in the *NIH Guidelines* when a *Major Action* is involved;

Section IV-C-1-b-(1)-(b). Assigning containment levels for types of experiments that are not explicitly considered in the *NIH Guidelines* when a *Major Action* is involved;

Section IV-C-1-b-(1)-(c). Promulgating and amending a list of classes of recombinant or synthetic nucleic acid molecules to be exempt from the *NIH Guidelines* because they consist entirely of DNA segments from species that exchange DNA by known physiological processes or otherwise do not present a significant risk to health or the environment;

Section IV-C-1-b-(1)-(d). Permitting experiments specified by Section III-A, *Experiments that Require Institutional Biosafety Committee Approval, RAC Review, and NIH Director Approval Before Initiation*;

Section IV-C-1-b-(1)-(e). Certifying new host-vector systems with the exception of minor modifications of already certified systems (the standards and procedures for certification are described in Appendix I-II, *Certification of Host-Vector Systems*). Minor modifications constitute (e.g., those of minimal or no consequence to the properties relevant to containment); and

Section IV-C-1-b-(1)-(f). Adopting other changes in the *NIH Guidelines*.

Section IV-C-1-b-(2). Minor Actions

NIH/OBA shall carry out certain functions as delegated to it by the NIH Director (see Section IV-C-3, *Office of Biotechnology Activities*). *Minor Actions* (as determined by NIH/OBA in consultation with the RAC Chair and one or more RAC members, as necessary) will be transmitted to RAC and Institutional Biosafety Committee Chairs:

Section IV-C-1-b-(2)-(a). Changing containment levels for experiments that are specified in Section III, *Experiments Covered by the NIH Guidelines* (except when a *Major Action* is involved);

Section IV-C-1-b-(2)-(b). Assigning containment levels for experiments not explicitly considered in the *NIH Guidelines*;

Section IV-C-1-b-(2)-(c). Revising the *Classification of Etiologic Agents* for the purpose of these *NIH Guidelines* (see Section V-A, *Footnotes and References of Sections I-IV*).

Section IV-C-1-b-(2)-(d). Interpreting the *NIH Guidelines* for experiments to which the *NIH Guidelines* do not specifically assign containment levels;

Section IV-C-1-b-(2)-(e). Setting containment under Sections III-D-1-d, *Experiments Using Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents as Host-Vector Systems*, and III-D-2-b, *Experiments in which DNA from Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents is Cloned into Nonpathogenic Prokaryotic or Lower Eukaryotic Host-Vector Systems*;

Section IV-C-1-b-(2)-(f). Approving minor modifications of already certified host-vector systems (the standards and procedures for such modifications are described in Appendix I-II, *Certification of Host-Vector Systems*);

Section IV-C-1-b-(2)-(g). Decertifying already certified host-vector systems;

Section IV-C-1-b-(2)-(h). Adding new entries to the list of molecules toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*); and

Section IV-C-1-b-(2)-(i). Determining appropriate containment conditions for experiments according to case precedents developed under Section IV-C-1-b-(2)-(c).

Section IV-C-2. Recombinant DNA Advisory Committee (RAC)

The RAC is responsible for carrying out the functions specified in the *NIH Guidelines*, as well as others specified in its charter or assigned by the Secretary of Health and Human Services or the NIH Director. The RAC membership and procedures, in addition to those set forth in the *NIH Guidelines*, are specified in the charter for the RAC which is filed as provided in the General Services Administration Federal Advisory Committee Management regulations, 41 CFR part 101-6, and is available on the OBA web site, http://oba.od.nih.gov/oba/rac/RACCharter2009.pdf. In the event of a conflict between the *NIH Guidelines* and the charter, the charter shall control.

The RAC will consist of not less than 15 voting members, including the Chair, appointed under the procedures of the NIH and the Department of Health and Human Services. The maximum number of voting members will be established in the charter of the RAC. At least a majority of the voting members must be knowledgeable in relevant scientific fields, e.g., molecular genetics, molecular biology, recombinant or synthetic nucleic acid molecule research, including clinical gene transfer research. At least 4 members of the RAC must be knowledgeable in fields such as public health, laboratory safety, occupational health, protection of human subjects of research, the environment, ethics, law, public attitudes or related fields. Representatives of the Federal agencies listed in the charter shall serve as non-voting members. Nominations for RAC members may be submitted to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

All meetings of the RAC shall be announced in the *Federal Register*, including tentative agenda items, 15 days before the meeting. Final agendas, if modified, shall be available at least 72 hours before the meeting. No item defined as a *Major Action* under Section IV-C-1-b-(1) may be added to an agenda following *Federal Register* publication.

RAC shall be responsible for:

Section IV-C-2-a. Advising the NIH Director on the following actions: (1) Adopting changes in the *NIH Guidelines*. (2) Assigning containment levels, changing containment levels, and approving experiments considered as *Major Actions* under the *NIH Guidelines*, i.e., the deliberate transfer of a drug resistance trait to microorganisms that are not known to acquire the trait naturally, if such acquisition could compromise the use of the drug to control disease agents in humans, veterinary medicine, or agriculture. (3) Promulgating and amending lists of classes of recombinant or synthetic nucleic acid molecules to be exempt from the *NIH Guidelines* because they consist entirely of DNA segments from species that exchange DNA by known physiological processes or otherwise do not present a significant risk to health or the environment. (4) Certifying new host-vector systems.

Section IV-C-2-b. Identifying novel human gene transfer experiments deserving of public discussion by the full RAC;

Section IV-C-2-c. Transmitting to the NIH Director specific comments/ recommendations about: (i) a specific human gene transfer experiment, or (ii) a category of human gene transfer experiments;

Section IV-C-2-d. Publicly reviewing human gene transfer clinical trial data and relevant information evaluated and summarized by NIH/OBA in accordance with the annual data reporting requirements;

Section IV-C-2-e. Identifying broad scientific, safety, social, and ethical issues relevant to gene therapy research as potential Gene Therapy Policy Conference topics;

Section IV-C-2-f. Identifying novel social and ethical issues relevant to specific human applications of gene transfer and recommending appropriate modifications to the *Points to Consider* that will provide guidance in the preparation of relevant Informed Consent documents; and

Section IV-C-2-g. Identifying novel scientific and safety issues relevant to specific human applications of gene transfer and recommending appropriate modifications to the *Points to Consider* that will provide guidance in the design and submission of human gene transfer clinical trials.

Section IV-C-3. Office of Biotechnology Activities (OBA)

OBA shall serve as a focal point for information on recombinant or synthetic nucleic acid molecule activities and provide advice to all within and outside NIH including institutions, Biological Safety Officers, Principal Investigators, Federal agencies, state and local governments, and institutions in the private sector. OBA shall carry out such other functions as may be delegated to it by the NIH Director. OBA's responsibilities include (but are not limited to) the following:

Section IV-C-3-a. Serving as the focal point for public access to summary information pertaining to human gene transfer experiments;

Section IV-C-3-b. Serving as the focal point for data management of human gene transfer experiments;

Section IV-C-3-c. Administering the annual data reporting requirements (and subsequent review) for human gene transfer experiments (see Appendix M-I-C, *Reporting Requirements*);

Section IV-C-3-d. Transmitting comments/recommendations arising from public RAC discussion of a novel human gene transfer experiment to the NIH Director. RAC recommendations shall be forwarded to the Principal Investigator, the sponsoring institution, and other DHHS components, as appropriate.

Section IV-C-3-e. Collaborating with Principal Investigators, Institutional Biosafety Committees, Institutional Review Boards, and other DHHS components (including FDA and the Office for Human Research Protections), to ensure human gene transfer experiment registration compliance in accordance with Appendix M-I, *Requirements for Protocol Submission, Review, and Reporting-Human Gene Transfer Experiments* of the *NIH Guidelines*.

Section IV-C-3-f. Administering Gene Therapy Policy Conferences as deemed appropriate by the NIH Director (see Appendix L, *Gene Therapy Policy Conferences*).

Section IV-C-3-g. Reviewing and approving experiments in conjunction with *ad hoc* experts involving the cloning of genes encoding for toxin molecules that are lethal for vertebrates at an LD_{50} of less than or equal to 100 nanograms per kilogram body weight in organisms other than *Escherichia coli* K-12 (see Section III-B-1, *Experiments Involving the Cloning of Toxin Molecules with LD_{50} of Less than 100 Nanograms Per Kilogram Body Weight*, Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*);

Section IV-C-3-h. Serving as the executive secretary of RAC;

Section IV-C-3-i. Publishing in the *Federal Register*:

Section IV-C-3-i-(1). Announcements of RAC meetings and tentative agendas at least 15 days in advance (Note: If the agenda for a RAC meeting is modified, OBA shall make the revised agenda available to anyone upon request in advance of the meeting);

Section IV-C-3-i-(2). Announcements of Gene Therapy Policy Conferences and tentative agendas at least 15 days in advance;

Section IV-C-3-i-(3). Proposed *Major Actions* (see Section IV-C-1-b-(1), *Major Actions*) at least 15 days prior to the RAC meeting; and

Section IV-C-3-j. Reviewing and approving the membership of an institution's Institutional Biosafety Committee, and where it finds the Institutional Biosafety Committee meets the requirements set forth in Section IV-B-2, *Institutional Biosafety Committee (IBC)*, giving its approval to the Institutional Biosafety Committee membership.

Section IV-C-4. Other NIH Components

Other NIH components shall be responsible for certifying maximum containment (BL4) facilities, inspecting them periodically, and inspecting other recombinant or synthetic nucleic acid molecule facilities as deemed necessary.

Section IV-D. Voluntary Compliance

Section IV-D-1. Basic Policy - Voluntary Compliance

Individuals, corporations, and institutions not otherwise covered by the *NIH Guidelines* are encouraged to follow the standards and procedures set forth in Sections I through IV. In order to simplify discussion, references hereafter to "institutions" are intended to encompass corporations and individuals who have no organizational affiliation. For purposes of complying with the *NIH Guidelines*, an individual intending to carry out research involving recombinant or synthetic nucleic acid molecules is encouraged to affiliate with an institution that has an Institutional Biosafety Committee approved under the *NIH Guidelines*.

Since commercial organizations have special concerns, such as protection of proprietary data, some modifications and explanations of the procedures are provided in Sections IV-D-2 through IV-D-5-b, *Voluntary Compliance*, in order to address these concerns.

Section IV-D-2. Institutional Biosafety Committee Approval - Voluntary Compliance

It should be emphasized that employment of an Institutional Biosafety Committee member solely for purposes of membership on the Institutional Biosafety Committee does not itself make the member an institutionally affiliated member. Except for the unaffiliated members, a member of an Institutional Biosafety Committee for an institution not otherwise covered by the *NIH Guidelines* may participate in the review and approval of a project in which the member has a direct financial interest so long as the member has not been, and does not expect to be, engaged in the project. Section IV-B-2-a-(4), *Institutional Biosafety Committee*, is modified to that extent for purposes of these institutions.

Section IV-D-3. Certification of Host-Vector Systems - Voluntary Compliance

A host-vector system may be proposed for certification by the NIH Director in accordance with the procedures set forth in Appendix I-II, *Certification of Host-Vector Systems*. In order to ensure protection for proprietary data, any public notice regarding a host-vector system which is designated by the institution as proprietary under Section IV-D, *Voluntary Compliance*, will be issued only after consultation with the institution as to the content of the notice.

Section IV-D-4. Requests for Exemptions and Approvals - Voluntary Compliance

Requests for exemptions or other approvals as required by the *NIH Guidelines* should be submitted based on the procedures set forth in Sections I through IV. In order to ensure protection for proprietary data, any public notice regarding a request for an exemption or other approval which is designated by the institution as

proprietary under Section IV-D-5-a, *Voluntary Compliance*, will be issued only after consultation with the institution as to the content of the notice.

Section IV-D-5. Protection of Proprietary Data - Voluntary Compliance

Section IV-D-5-a. General

In general, the Freedom of Information Act requires Federal agencies to make their records available to the public upon request. However, this requirement does not apply to, among other things, "trade secrets and commercial or financial information that is obtained from a person and that is privileged or confidential." Under 18 U.S.C. 1905, it is a criminal offense for an officer or employee of the U.S. or any Federal department or agency to publish, divulge, disclose, or make known "in any manner or to any extent not authorized by law any information coming to him in the course of his employment or official duties or by reason of any examination or investigation made by, or return, report or record made to or filed with, such department or agency or officer or employee thereof, which information concerns or relates to the trade secrets, (or) processes...of any person, firm, partnership, corporation, or association." This provision applies to all employees of the Federal Government, including special Government employees. Members of RAC are "special Government employees."

In submitting to NIH for purposes of voluntary compliance with the *NIH Guidelines*, an institution may designate those items of information which the institution believes constitute trade secrets, privileged, confidential, commercial, or financial information. If NIH receives a request under the Freedom of Information Act for information so designated, NIH will promptly contact the institution to secure its views as to whether the information (or some portion) should be released. If NIH decides to release this information (or some portion) in response to a Freedom of Information request or otherwise, the institution will be advised and the actual release will be delayed in accordance with 45 Code of Federal Regulations, Section 5.65(d) and (e).

Section IV-D-5-b. Pre-submission Review

Any institution not otherwise covered by the *NIH Guidelines*, which is considering submission of data or information voluntarily to NIH, may request pre-submission review of the records involved to determine if NIH will make all or part of the records available upon request under the Freedom of Information Act.

A request for pre-submission review should be submitted to NIH/OBA along with the records involved to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). These records shall be clearly marked as being the property of the institution on loan to NIH solely for the purpose of making a determination under the Freedom of Information Act. NIH/OBA will seek a determination from the responsible official under DHHS regulations (45 CFR Part 5) as to whether the records involved, (or some portion) will be made available to members of the public under the Freedom of Information Act. Pending such a determination, the records will be kept separate from NIH/OBA files, will be considered records of the institution and not NIH/OBA, and will not be received as part of NIH/OBA files. No copies will be made of such records.

NIH/OBA will inform the institution of the NIH Freedom of Information Officer's determination and follow the institution's instructions as to whether some or all of the records involved are to be returned to the institution or to become a part of NIH/OBA files. If the institution instructs NIH/OBA to return the records, no copies or summaries of the records will be made or retained by DHHS, NIH, or OBA. The NIH Freedom of Information Officer's determination will represent that official's judgment at the time of the determination as to whether the records involved (or some portion) would be exempt from disclosure under the Freedom of Information Act if at the time of the determination the records were in NIH/OBA files and a request was received for such files under the Freedom of Information Act.

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SECTION V. FOOTNOTES AND REFERENCES OF SECTIONS I THROUGH IV

Section V-A. The NIH Director, with advice of the RAC, may revise the classification for the purposes of the *NIH Guidelines* (see Section IV-C-1-b-(2)-(e), *Minor Actions*). The revised list of organisms in each Risk Group is reprinted in Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*.

Section V-B. Section III, *Experiments Covered by the NIH Guidelines*, describes a number of places where judgments are to be made. In all these cases, the Principal Investigator shall make the judgment on these matters as part of his/her responsibility to "make the initial determination of the required levels of physical and biological containment in accordance with the *NIH Guidelines*" (see Section IV-B-7-c-(1)). For cases falling under Sections III-A through III-E, *Experiments Covered by the NIH Guidelines*, this judgment is to be reviewed and approved by the Institutional Biosafety Committee as part of its responsibility to make an "independent assessment of the containment levels required by the *NIH Guidelines* for the proposed research" (see Section IV-B-2-b-(1), *Institutional Biosafety Committee*). The Institutional Biosafety Committee may refer specific cases to NIH/OBA as part of NIH/OBA's functions to "provide advice to all within and outside NIH" (see Section IV-C-3). NIH/OBA may request advice from the RAC as part of the RAC's responsibility for "interpreting the *NIH Guidelines* for experiments to which the *NIH Guidelines* do not specifically assign containment levels" (see Section IV-C-1-b-(2)-(f), *Minor Actions*).

Section V-C. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and the National Institutes of Health. *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition, 2007. Copies are available from: Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20401-0001, Phone (202) 512-1800 [http://www.gpo.gov/].

Section V-D. *Classification of Etiologic Agents on the Basis of Hazard*, 4th Edition, July 1974, U.S. Department of Health, Education, and Welfare, Public Health Service, Centers for Disease Control, Office of Biosafety, Atlanta, Georgia 30333.

Section V-E. Chin, James ed., *Control of Communicable Diseases Manual*, 17th Edition, 2000. ISBN: 087553-242-X, American Public Health Association, 800 I Street, N.W., Washington, D.C. Phone: (202) 777-2742.

Section V-F. *World Health Organization Laboratory Biosafety Manual*, 2nd edition. 1993. WHO Albany, NY. Copies are available from: WHO Publication Centre, USA, (Q Corp) 49 Sheridan Avenue, Albany, New York 12210; Phone: (518) 436-9686 (Order # 1152213).

Section V-G. A U.S. Department of Agriculture permit, required for import and interstate transport of plant and animal pathogens, may be obtained from the U.S. Department of Agriculture, ATTN: Animal and Plant Health Inspection Service (APHIS), Veterinary Services, National Center for Import-Export, Products Program, 4700 River Road, Unit 40, Riverdale, Maryland 20737. Phone: (301) 734-8499; Fax: (301) 734-8226.

Section V-H. American Type Culture Collection Catalogues of plant viruses, animal viruses, cells, bacteria, fungi, etc. are available from American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209. Phone: (703) 365-2700.

Section V-I. U.S. Department of Labor, Occupational Safety and Health Administration, 29 CFR 1910.1030, *Bloodborne Pathogens*. See also, *Exposure to Bloodborne Pathogens,* OSHA 3127, 1996 (Revised).

Section V-J. As classified in the *Virus Taxonomy: The Classification and Nomenclature of Viruses. The Seventh Report of the International Committee on Taxonomy of Viruses*, Academic Press, 2000 (0123702003) San Diego, CA.

Section V-K. *i.e.*, the total of all genomes within a family shall not exceed two-thirds of the genome.

Section V-L. Organisms including alastrim, smallpox (variola) and whitepox may not be studied in the United States except at specified facilities. All activities, including storage of variola and whitepox, are restricted to the single national facility (World Health Organization Collaborating Center for Smallpox Research, Centers for Disease Control and Prevention, Atlanta, Georgia).

Section V-M. In accordance with accepted scientific and regulatory practices of the discipline of plant pathology, an exotic plant pathogen (e.g., virus, bacteria, or fungus) is one that is unknown to occur within the U.S. (see Section V-G, *Footnotes and References of Sections I-IV*). Determination of whether a pathogen has a potential for serious detrimental impact on managed (agricultural, forest, grassland) or natural ecosystems should be made by the Principal Investigator and the Institutional Biosafety Committee, in consultation with scientists knowledgeable of plant diseases, crops, and ecosystems in the geographic area of the research.

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APPENDIX A. EXEMPTIONS UNDER SECTION III-F-6--SUBLISTS OF NATURAL EXCHANGERS

Certain specified recombinant or synthetic nucleic acid molecules that consist entirely of DNA segments from different species that exchange DNA by known physiological processes, though one or more of the segments may be a synthetic equivalent are exempt from these *NIH Guidelines* (see Section III-F-6, *Exempt Experiments*). Institutional Biosafety Committee registration is not required for these exempt experiments. A list of such exchangers will be prepared and periodically revised by the NIH Director with advice from the RAC after appropriate notice and opportunity for public comment (see Section IV-C-1-b-(1)-(c), *NIH Director--Specific Responsibilities*). For a list of natural exchangers that are exempt from the *NIH Guidelines*, see Appendices A-I through A-VI, *Exemptions under Section III-F-6 Sublists of Natural Exchangers.* Section III-F-6, *Exempt Experiments*, describes recombinant or synthetic nucleic acid molecules that are: (1) composed entirely of DNA segments from one or more of the organisms within a sublist, and (2) to be propagated in any of the organisms within a sublist (see *Classification of Bergey's Manual of Determinative Bacteriology*; 8th edition, R. E. Buchanan and N. E. Gibbons, editors, Williams and Wilkins Company; Baltimore, Maryland 1984). Although these experiments are exempt, it is recommended that they be performed at the appropriate biosafety level for the host or recombinant/synthetic organism (see *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition, 2007, U.S. DHHS, Public Health Service, Centers for Disease Control and Prevention, Atlanta, Georgia, and NIH Office of Biosafety, Bethesda, Maryland).

Appendix A-I. Sublist A

Genus Escherichia
Genus *Shigella*
Genus *Salmonella* - including *Arizona*
Genus *Enterobacter*
Genus *Citrobacter* - including *Levinea*
Genus *Klebsiella* - including *oxytoca*
Genus *Erwinia*
Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas fluorescens, and Pseudomonas mendocina
Serratia marcescens
Yersinia enterocolitica

Appendix A-II. Sublist B

Bacillus subtilis
Bacillus licheniformis
Bacillus pumilus
Bacillus globigii
Bacillus niger
Bacillus nato
Bacillus amyloliquefaciens
Bacillus aterrimus

Appendix A-III. Sublist C

Streptomyces aureofaciens
Streptomyces rimosus
Streptomyces coelicolor

Appendix A-IV. Sublist D

Streptomyces griseus
Streptomyces cyaneus
Streptomyces venezuelae

Appendix A-V. Sublist E

One way transfer of *Streptococcus mutans* or *Streptococcus lactis* DNA into *Streptococcus sanguis*

Appendix A-VI. Sublist F

Streptococcus sanguis
Streptococcus pneumoniae
Streptococcus faecalis
Streptococcus pyogenes
Streptococcus mutans

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APPENDIX B. CLASSIFICATION OF HUMAN ETIOLOGIC AGENTS ON THE BASIS OF HAZARD

This appendix includes those biological agents known to infect humans as well as selected animal agents that may pose theoretical risks if inoculated into humans. Included are lists of representative genera and species known to be pathogenic; mutated, recombined, and non-pathogenic species and strains are not considered. Non-infectious life cycle stages of parasites are excluded.

This appendix reflects the current state of knowledge and should be considered a resource document. Included are the more commonly encountered agents and is not meant to be all-inclusive. Information on agent risk assessment may be found in the *Agent Summary Statements* of the CDC/NIH publication, *Biosafety in Microbiological and Biomedical Laboratories* (see Sections V-C, V-D, V-E, and V-F, *Footnotes and References of Sections I through IV*. Further guidance on agents not listed in Appendix B may be obtained through: Centers for Disease Control and Prevention, Biosafety Branch, Atlanta, Georgia 30333, Phone: (404) 639-3883, Fax: (404) 639-2294; National Institutes of Health, Division of Safety, Bethesda, Maryland 20892, Phone: (301) 496-1357; National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa 50010, Phone: (515) 862-8258.

A special committee of the American Society for Microbiology will conduct an annual review of this appendix and its recommendation for changes will be presented to the Recombinant DNA Advisory Committee as proposed amendments to the *NIH Guidelines*.

Appendix B - Table 1. Basis for the Classification of Biohazardous Agents by Risk Group (RG)

Risk Group 1 (RG1)	Agents that are not associated with disease in healthy adult humans
Risk Group 2 (RG2)	Agents that are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are *often* available
Risk Group 3 (RG3)	Agents that are associated with serious or lethal human disease for which preventive or therapeutic interventions *may be* available (high individual risk but low community risk)
Risk Group 4 (RG4)	Agents that are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are *not usually* available (high individual risk and high community risk)

Appendix B-I. Risk Group 1 (RG1) Agents

RG1 agents are not associated with disease in healthy adult humans. Examples of RG1 agents include asporogenic *Bacillus subtilis* or *Bacillus licheniformis* (see Appendix C-IV-A, *Bacillus subtilis* or *Bacillus licheniformis* Host-Vector Systems, Exceptions); adeno- associated virus (AAV – all serotypes); and recombinant or synthetic AAV constructs, in which the transgene does not encode either a potentially tumorigenic gene product or a toxin molecule and are produced in the absence of a helper virus. A strain of *Escherichia coli* (see Appendix C-II-A, *Escherichia coli* K-12 Host Vector Systems, Exceptions) is an RG1 agent if it (1) does not possess a complete lipopolysaccharide (*i.e.*, lacks the O antigen); and (2) does not carry any active virulence factor (*e.g.*, toxins) or colonization factors and does not carry any genes encoding these factors.

Those agents not listed in Risk Groups (RGs) 2, 3 and 4 are not automatically or implicitly classified in RG1; a risk assessment must be conducted based on the known and potential properties of the agents and their relationship to agents that are listed.

Appendix B-II. Risk Group 2 (RG2) Agents

RG2 agents are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are *often* available.

Appendix B-II-A. Risk Group 2 (RG2) - Bacterial Agents Including Chlamydia

--*Acinetobacter baumannii* (formerly *Acinetobacter calcoaceticus*)
--*Actinobacillus*
--*Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*)
--*Aeromonas hydrophila*
--*Amycolata autotrophica*
--*Archanobacterium haemolyticum* (formerly *Corynebacterium haemolyticum*)
--*Arizona hinshawii* - all serotypes
--*Bacillus anthracis*
--*Bartonella henselae, B. quintana, B. vinsonii*
--*Bordetella* including *B. pertussis*
--*Borrelia recurrentis, B. burgdorferi*
--*Burkholderia* (formerly *Pseudomonas* species) except those listed in Appendix B-III-A (RG3))
--*Campylobacter coli, C. fetus, C. jejuni*
--*Chlamydia psittaci, C. trachomatis, C. pneumoniae*
--*Clostridium botulinum, C. chauvoei, C. haemolyticum, C. histolyticum, C. novyi, C. septicum, C. tetani*
--*Coxiella burnetii* – specifically the Phase II, Nine Mile strain, plaque purified, clone 4
--*Corynebacterium diphtheriae, C. pseudotuberculosis, C. renale*
--*Dermatophilus congolensis*
--*Edwardsiella tarda*
--*Erysipelothrix rhusiopathiae*
--*Escherichia coli* - all enteropathogenic, enterotoxigenic, enteroinvasive and strains bearing K1 antigen, including *E. coli* O157:H7
--*[*]Francisella tularensis* specifically *[*]F. tualrensis* subspecies *novocida* [aka *F. novocida*], strain Utah 112; *[*]F. tularensis* subspecies *holartica* LVS; *[*]F tularensis* biovar *tularensis* strain ATCC 6223 (aka strain B38)
--*Haemophilus ducreyi, H. influenzae*
--*Helicobacter pylori*
--*Klebsiella* - all species except *K. oxytoca* (RG1)
--*Legionella* including *L. pneumophila*
--*Leptospira interrogans* - all serotypes
--*Listeria*
--*Moraxella*
--*Mycobacterium* (except those listed in Appendix B-III-A (RG3)) including *M. avium* complex, *M. asiaticum, M. bovis* BCG vaccine strain, *M. chelonei, M. fortuitum, M. kansasii, M. leprae, M. malmoense, M. marinum, M. paratuberculosis, M. scrofulaceum, M. simiae, M. szulgai, M. ulcerans, M. xenopi*
--*Mycoplasma*, except *M. mycoides* and *M. agalactiae* which are restricted animal pathogens
--*Neisseria gonorrhoeae, N. meningitidis*
--*Nocardia asteroides, N. brasiliensis, N. otitidiscaviarum, N. transvalensis*
--*Rhodococcus equi*
--*Salmonella* including *S. arizonae, S. cholerasuis, S. enteritidis, S. gallinarum-pullorum, S. meleagridis, S. paratyphi*, A, B, C, *S. typhi, S. typhimurium*
--*Shigella* including *S. boydii, S. dysenteriae*, type 1, *S. flexneri, S. sonnei*
--*Sphaerophorus necrophorus*
--*Staphylococcus aureus*
--*Streptobacillus moniliformis*

[*] For research involving high concentrations, BL3 practices should be considered (See Appendix G-II-C-2. Special Practices (BL3)).

--*Streptococcus* including *S. pneumoniae, S. pyogenes*
--*Treponema pallidum, T. carateum*
--*Vibrio cholerae, V. parahemolyticus, V. vulnificus*
--*Yersinia enterocolitica*
--*Yersinia pestis* specifically $pgm^{(-)}$ strains (lacking the 102 kb pigmentation locus) and $lcr^{(-)}$ strains (lacking the LCR plasmid)

Appendix B-II-B. Risk Group 2 (RG2) - Fungal Agents

--*Blastomyces dermatitidis*
--*Cladosporium bantianum, C. (Xylohypha) trichoides*
--*Cryptococcus neoformans*
--*Dactylaria galopava (Ochroconis gallopavum)*
--*Epidermophyton*
--*Exophiala (Wangiella) dermatitidis*
--*Fonsecaea pedrosoi*
--*Microsporum*
--*Paracoccidioides braziliensis*
--*Penicillium marneffei*
--*Sporothrix schenckii*
--*Trichophyton*

Appendix B-II-C. Risk Group 2 (RG2) - Parasitic Agents

--*Ancylostoma* human hookworms including *A. duodenale, A. ceylanicum*
--*Ascaris* including *Ascaris lumbricoides suum*
--*Babesia* including *B. divergens, B. microti*
--*Brugia* filaria worms including *B. malayi, B. timori*
--*Coccidia*
--*Cryptosporidium* including *C. parvum*
--*Cysticercus cellulosae* (hydatid cyst, larva of *T. solium*)
--*Echinococcus* including *E. granulosis, E. multilocularis, E. vogeli*
--*Entamoeba histolytica*
--*Enterobius*
--*Fasciola* including *F. gigantica, F. hepatica*
--*Giardia* including *G. lamblia*
--*Heterophyes*
--*Hymenolepis* including *H. diminuta, H. nana*
--*Isospora*
--*Leishmania* including *L. braziliensis, L. donovani, L. ethiopia, L. major, L. mexicana, L. peruvania, L. tropica*
--*Loa loa* filaria worms
--*Microsporidium*
--*Naegleria fowleri*
--*Necator* human hookworms including *N. americanus*
--*Onchocerca* filaria worms including, *O. volvulus*
--*Plasmodium* including simian species, *P. cynomologi, P. falciparum, P. malariae, P. ovale, P. vivax*
--*Sarcocystis* including *S. sui hominis*
--*Schistosoma* including *S. haematobium, S. intercalatum, S. japonicum, S. mansoni, S. mekongi*
--*Strongyloides* including *S. stercoralis*
--*Taenia solium*
--*Toxocara* including *T. canis*
--*Toxoplasma* including *T. gondii*
--*Trichinella spiralis*
--*Trypanosoma* including *T. brucei brucei, T. brucei gambiense, T. brucei rhodesiense, T. cruzi*
--*Wuchereria bancrofti* filaria worms

Appendix B-II-D. Risk Group 2 (RG2) - Viruses

Adenoviruses, human - all types

Alphaviruses (Togaviruses) - Group A Arboviruses
--Chikungunya vaccine strain 181/25
--Eastern equine encephalomyelitis virus
--Venezuelan equine encephalomyelitis vaccine strains TC-83 and V3526
--Western equine encephalomyelitis virus

Arenaviruses
--Junin virus candid #1 vaccine strain
--Lymphocytic choriomeningitis virus (non-neurotropic strains)
--Tacaribe virus complex
--Other viruses as listed in the reference source (see Section V-C, *Footnotes and References of Sections I through IV*)

Bunyaviruses
--Bunyamwera virus
--Rift Valley fever virus vaccine strain MP-12
--Other viruses as listed in the reference source (see Section V-C, *Footnotes and References of Sections I through IV*)

Caliciviruses

Coronaviruses

Flaviviruses - Group B Arboviruses
--Dengue virus serotypes 1, 2, 3, and 4
--Japanese encephalitis virus strain SA 14-14-2
--Yellow fever virus vaccine strain 17D
--Other viruses as listed in the reference source (see Section V-C, *Footnotes and References of Sections I through IV*)

Hepatitis A, B, C, D, and E viruses

Herpesviruses - except Herpesvirus simiae (Monkey B virus) (see Appendix B-IV-D, *Risk Group 4 (RG4) - Viral Agents*)
--Cytomegalovirus
--Epstein Barr virus
--*Herpes simplex* types 1 and 2
--*Herpes zoster*
--Human herpesvirus types 6 and 7

Orthomyxoviruses
--Influenza viruses types A, B, and C (except those listed in Appendix B-III-D, *Risk Group 3 (RG3) - Viruses and Prions*)
--Tick-borne orthomyxoviruses

Papilloma viruses
--All human papilloma viruses

Paramyxoviruses
--Newcastle disease virus
--Measles virus
--Mumps virus
--Parainfluenza viruses types 1, 2, 3, and 4
--Respiratory syncytial virus

Parvoviruses
--Human parvovirus (B19)

Picornaviruses
--Coxsackie viruses types A and B
--Echoviruses - all types
--Polioviruses - all types, wild and attenuated
--Rhinoviruses - all types

Poxviruses - all types except Monkeypox virus (see Appendix B-III-D, *Risk Group 3 (RG3) - Viruses and Prions*) and restricted poxviruses including Alastrim, Smallpox, and Whitepox (see Section V-L, *Footnotes and References of Sections I through IV*)

Reoviruses - all types including Coltivirus, human Rotavirus, and Orbivirus (Colorado tick fever virus)

Rhabdoviruses
--Rabies virus - all strains
--Vesicular stomatitis virus non exotic strains: VSV-Indiana 1 serotype strains (*e.g.* Glasgow, Mudd-Summers, Orsay, San Juan) and VSV-New Jersey serotype strains (*e.g.* Ogden, Hazelhurst)

Rubivirus (Togaviruses)
--Rubella virus

Appendix B-III. Risk Group 3 (RG3) Agents

RG3 agents are associated with serious or lethal human disease for which preventive or therapeutic interventions *may be* available.

Appendix B-III-A. Risk Group 3 (RG3) - Bacterial Agents Including Rickettsia

--*Bartonella*
--*Brucella* including *B. abortus, B. canis, B. suis*
--*Burkholderia (Pseudomonas) mallei, B. pseudomallei*
--*Coxiella burnetii* (except the Phase II, Nine Mile strain listed in Appendix B-II-A, *Risk Group 2 (RG2) - Bacterial Agents Including Chlamydia)*
--*Francisella tularensis* (except those strains listed in Appendix B-II-A, *Risk Group 2 (RG2) - Bacterial Agents Including Chlamydia)*
--*Mycobacterium bovis* (except BCG strain, see Appendix B-II-A, *Risk Group 2 (RG2) - Bacterial Agents Including Chlamydia*), *M. tuberculosis*
--*Pasteurella multocida* type B -"buffalo" and other virulent strains
--Rickettsia akari, R. australis, R. canada, R. conorii, R. prowazekii, R. rickettsii, R, siberica, R. tsutsugamushi, R. typhi (R. mooseri)
--*Yersinia pestis* (except those strains listed in Appendix B-II-A, *Risk Group 2 (RG2) - Bacterial Agents Including Chlamydia)*

Appendix B-III-B. Risk Group 3 (RG3) - Fungal Agents

--*Coccidioides immitis* (sporulating cultures; contaminated soil)
--*Histoplasma capsulatum, H. capsulatum var. duboisii*

Appendix B-III-C. Risk Group 3 (RG3) - Parasitic Agents

None

Appendix B-III-D. Risk Group 3 (RG3) - Viruses and Prions

Alphaviruses (Togaviruses) - Group A Arboviruses
--Chikungunya virus (except the vaccine strain 181/25 listed in Appendix B-II-D Risk Group2 (RG2) – Viruses)
--Semliki Forest virus
--St. Louis encephalitis virus
--Venezuelan equine encephalomyelitis virus (except the vaccine strains TC-83 and V3526, see Appendix B-II-D (RG2) – Viruses)
--Other viruses as listed in the reference source (see Section V-C, *Footnotes and References of Sections I through IV*)

Arenaviruses
--Flexal
--Lymphocytic choriomeningitis virus (LCM) (neurotropic strains)

Bunyaviruses
--Hantaviruses including Hantaan virus
--Rift Valley fever virus

Coronaviruses
--SARS-associated coronavirus (SARS-CoV)

Flaviviruses - Group B Arboviruses
--Japanese encephalitis virus (except those strains listed in Appendix B-II-D Risk Group2 (RG2) - Viruses)
--West Nile virus (WNV)
--Yellow fever virus
--Other viruses as listed in the reference source (see Section V-C, *Footnotes and References of Sections I through IV*)

Orthomyxoviruses
-- Influenza viruses 1918-1919 H1N1 (1918 H1N1), human H2N2 (1957-1968), and highly pathogenic avian influenza H5N1 strains within the Goose/Guangdong/96-like H5 lineage (HPAI H5N1).

Poxviruses
--Monkeypox virus

Prions
--Transmissible spongioform encephalopathies (TME) agents (Creutzfeldt-Jacob disease and kuru agents)(see Section V-C, *Footnotes and References of Sections I through IV*, for containment instruction)

Retroviruses
--Human immunodeficiency virus (HIV) types 1 and 2
--Human T cell lymphotropic virus (HTLV) types 1 and 2
--Simian immunodeficiency virus (SIV)

Rhabdoviruses
--Vesicular stomatitis virus (except those strains listed in Appendix B-II-D Risk Group2 (RG2) - Viruses)

Appendix B-IV. Risk Group 4 (RG4) Agents

RG4 agents are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are *not usually* available.

Appendix B-IV-A. Risk Group 4 (RG4) - Bacterial Agents

None

Appendix B-IV-B. Risk Group 4 (RG4) - Fungal Agents

None

Appendix B-IV-C. Risk Group 4 (RG4) - Parasitic Agents

None

Appendix B-IV-D. Risk Group 4 (RG4) - Viral Agents

Arenaviruses
--Guanarito virus
--Lassa virus

--Junin virus (except the candid #1 vaccine strain listed in Appendix B-II-D Risk Group2 (RG2) – Viruses)
--Machupo virus
--Sabia

Bunyaviruses (Nairovirus)
--Crimean-Congo hemorrhagic fever virus

Filoviruses
--Ebola virus
--Marburg virus

Flaviruses - Group B Arboviruses
--Tick-borne encephalitis virus complex including Absetterov, Central European encephalitis, Hanzalova, Hypr, Kumlinge, Kyasanur Forest disease, Omsk hemorrhagic fever, and Russian spring-summer encephalitis viruses

Herpesviruses (alpha)
--Herpesvirus simiae (Herpes B or Monkey B virus)

Paramyxoviruses
--Equine morbillivirus

Hemorrhagic fever agents and viruses as yet undefined

Appendix B-V. Animal Viral Etiologic Agents in Common Use

The following list of animal etiologic agents is appended to the list of human etiologic agents. None of these agents is associated with disease in healthy adult humans; they are commonly used in laboratory experimental work.

A containment level appropriate for RG1 human agents is recommended for their use. For agents that are infectious to human cells, e.g., amphotropic and xenotropic strains of murine leukemia virus, a containment level appropriate for RG2 human agents is recommended.

Baculoviruses

Herpesviruses
--Herpesvirus ateles
--Herpesvirus saimiri
--Marek's disease virus
--Murine cytomegalovirus

Papilloma viruses
--Bovine papilloma virus
--Shope papilloma virus

Polyoma viruses
--Polyoma virus
--Simian virus 40 (SV40)

Retroviruses
--Avian leukosis virus
--Avian sarcoma virus
--Bovine leukemia virus
--Feline leukemia virus
--Feline sarcoma virus
--Gibbon leukemia virus
--Mason-Pfizer monkey virus
--Mouse mammary tumor virus
--Murine leukemia virus

--Murine sarcoma virus
--Rat leukemia virus

Appendix B-V-1. Murine Retroviral Vectors

Murine retroviral vectors to be used for human transfer experiments (less than 10 liters) that contain less than 50% of their respective parental viral genome and that have been demonstrated to be free of detectable replication competent retrovirus can be maintained, handled, and administered, under BL1 containment.

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APPENDIX C. EXEMPTIONS UNDER SECTION III-F-8

Section III-F-8 states that exempt from these *NIH Guidelines* are "those that do not present a significant risk to health or the environment (see Section IV-C-1-b-(1)-(c), *NIH Director--Specific Responsibilities*), as determined by the NIH Director, with the advice of the RAC, and following appropriate notice and opportunity for public comment. See Appendix C, *Exemptions under Sections III-F-8*, for other classes of experiments which are exempt from the *NIH Guidelines*." The following classes of experiments are exempt under Section III-F-8:

Appendix C-I. Recombinant or Synthetic Nucleic Acid Molecules in Tissue Culture

Recombinant or synthetic nucleic acid molecules containing less than one-half of any eukaryotic viral genome (all viruses from a single family being considered identical -- see Appendix C-VIII-E, *Footnotes and References of Appendix C*), that are propagated and maintained in cells in tissue culture are exempt from these *NIH Guidelines* with the exceptions listed in Appendix C-I-A.

Appendix C-I-A. Exceptions

The following categories are not exempt from the *NIH Guidelines*: (i) experiments described in Section III-B which require NIH/OBA and Institutional Biosafety Committee approval before initiation, (ii) experiments involving DNA from Risk Groups 3, 4, or restricted organisms (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) or cells known to be infected with these agents, (iii) experiments involving the deliberate introduction of genes coding for the biosynthesis of molecules that are toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*), and (iv) whole plants regenerated from plant cells and tissue cultures are covered by the exemption provided they remain axenic cultures even though they differentiate into embryonic tissue and regenerate into plantlets.

Appendix C-II. *Escherichia coli K-12* Host-Vector Systems

Experiments which use *Escherichia coli* K-12 host-vector systems, with the exception of those experiments listed in Appendix C-II-A, are exempt from the *NIH Guidelines* provided that: (i) the *Escherichia coli* host does not contain conjugation proficient plasmids or generalized transducing phages; or (ii) lambda or lambdoid or Ff bacteriophages or non-conjugative plasmids (see Appendix C-VIII-B, *Footnotes and References of Appendix C*) shall be used as vectors. However, experiments involving the insertion into *Escherichia coli* K-12 of DNA from

prokaryotes that exchange genetic information (see Appendix C-VIII-C, *Footnotes and References of Appendix C*) with *Escherichia coli* may be performed with any *Escherichia coli* K-12 vector (e.g., conjugative plasmid). When a non-conjugative vector is used, the *Escherichia coli* K-12 host may contain conjugation-proficient plasmids either autonomous or integrated, or generalized transducing phages. For these exempt laboratory experiments, Biosafety Level (BL) 1 physical containment conditions are recommended. For large-scale fermentation experiments, the appropriate physical containment conditions need be no greater than those for the host organism unmodified by recombinant or synthetic nucleic acid molecule techniques; the Institutional Biosafety Committee can specify higher containment if deemed necessary.

Appendix C-II-A. Exceptions

The following categories are not exempt from the *NIH Guidelines*: (i) experiments described in Section III-B which require NIH/OBA and Institutional Biosafety Committee approval before initiation, (ii) experiments involving DNA from Risk Groups 3, 4, or restricted organisms (see Appendix B, *Classification of Human*

Etiologic Agents on the Basis of Hazard, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) or cells known to be infected with these agents may be conducted under containment conditions specified in Section III-D-2 with prior Institutional Biosafety Committee review and approval, (iii) large-scale experiments (e.g., more than 10 liters of culture), and (iv) experiments involving the cloning of toxin molecule genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*).

Appendix C-III. *Saccharomyces* **Host-Vector Systems**

Experiments involving *Saccharomyces cerevisiae* and *Saccharomyces uvarum* host-vector systems, with the exception of experiments listed in Appendix C-III-A, are exempt from the *NIH Guidelines*. For these exempt experiments, BL1 physical containment is recommended. For large-scale fermentation experiments, the appropriate physical containment conditions need be no greater than those for the unmodified host organism; the Institutional Biosafety Committee can specify higher containment if deemed necessary.

Appendix C-III-A. Exceptions

The following categories are not exempt from the *NIH Guidelines*: (i) experiments described in Section III-B which require NIH/OBA and Institutional Biosafety Committee approval before initiation, (ii) experiments involving DNA from Risk Groups 3, 4, or restricted organisms (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) or cells known to be infected with these agents may be conducted under containment conditions specified in Section III-D-2 with prior Institutional Biosafety Committee review and approval, (iii) large-scale experiments (e.g., more than 10 liters of culture), and (iv) experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*).

Appendix C-IV. *Kluyveromyces* **Host-Vector Systems**

Experiments involving *Kluyveromyces lactis* host-vector systems, with the exception of experiments listed in Appendix C-IV-A, are exempt from the *NIH Guidelines* provided laboratory-adapted strains are used (i.e. strains that have been adapted to growth under optimal or defined laboratory conditions). For these exempt experiments, BL1 physical containment is recommended. For large-scale fermentation experiments, the appropriate physical containment conditions need be no greater than those for the unmodified host organism; the Institutional Biosafety Committee may specify higher containment if deemed necessary.

Appendix C-IV-A Exceptions

The following categories are not exempt from the *NIH Guidelines*: (i) experiments described in Section III-B, which require NIH/OBA and Institutional Biosafety Committee approval before initiation; (ii) experiments involving DNA from Risk Groups 3, 4, or restricted organisms (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) or cells known to be infected with these agents may be conducted under containment conditions specified in Section III-D-2 with prior Institutional Biosafety Committee review and approval; (iii) large-scale experiments (e.g., more than 10 liters of culture), and (v) experiments involving the deliberate cloning of genes

coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*).

Appendix C-V. *Bacillus subtilis* **or** *Bacillus licheniformis* **Host-Vector Systems**

Any asporogenic *Bacillus subtilis* or asporogenic *Bacillus licheniformis* strain which does not revert to a spore-former with a frequency greater than 10^{-7} may be used for cloning DNA with the exception of those experiments listed in Appendix C-V-A, *Exceptions*. For these exempt laboratory experiments, BL1 physical containment conditions are recommended. For large-scale fermentation experiments, the appropriate physical containment conditions need be no greater than those for the unmodified host organism; the Institutional Biosafety Committee can specify higher containment if it deems necessary.

Appendix C-V-A. Exceptions

The following categories are not exempt from the *NIH Guidelines*: (i) experiments described in Section III-B which require NIH/OBA and Institutional Biosafety Committee approval before initiation, (ii) experiments involving DNA from Risk Groups 3, 4, or restricted organisms (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) or cells known to be infected with these agents may be conducted under containment conditions specified in Section III-D-2 with prior Institutional Biosafety Committee review and approval, (iii) large-scale experiments (e.g., more than 10 liters of culture), and (iv) experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*).

Appendix C-VI. Extrachromosomal Elements of Gram Positive Organisms

Recombinant or synthetic nucleic acid molecules derived entirely from extrachromosomal elements of the organisms listed below (including shuttle vectors constructed from vectors described in Appendix C), propagated and maintained in organisms listed below are exempt from these *NIH Guidelines*.

Bacillus amyloliquefaciens
Bacillus amylosachariticus
Bacillus anthracis
Bacillus aterrimus
Bacillus brevis
Bacillus cereus
Bacillus globigii
Bacillus licheniformis
Bacillus megaterium
Bacillus natto
Bacillus niger
Bacillus pumilus
Bacillus sphaericus
Bacillus stearothermophilis
Bacillus subtilis
Bacillus thuringiensis
Clostridium acetobutylicum
Lactobacillus casei
Listeria grayi
Listeria monocytogenes
Listeria murrayi
Pediococcus acidilactici
Pediococcus damnosus
Pediococcus pentosaceus
Staphylococcus aureus
Staphylococcus carnosus
Staphylococcus epidermidis
Streptococcus agalactiae
Streptococcus anginosus

Streptococcus avium
Streptococcus cremoris
Streptococcus dorans
Streptococcus equisimilis
Streptococcus faecalis
Streptococcus ferus
Streptococcus lactis
Streptococcus ferns
Streptococcus mitior
Streptococcus mutans
Streptococcus pneumoniae
Streptococcus pyogenes

Streptococcus salivarious
Streptococcus sanguis
Streptococcus sobrinus
Streptococcus thermophylus

Appendix C-VI-A. Exceptions

The following categories are not exempt from the *NIH Guidelines*: (i) experiments described in Section III-B which require NIH/OBA and Institutional Biosafety Committee approval before initiation, (ii) experiments involving DNA from Risk Groups 3, 4, or restricted organisms (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) or cells known to be infected with these agents may be conducted under containment conditions specified in Section III-D-2 with prior Institutional Biosafety Committee review and approval, (iii) large-scale experiments (e.g., more than 10 liters of culture), and (iv) experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F, *Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates*).

Appendix C-VII. The Purchase or Transfer of Transgenic Rodents

The purchase or transfer of transgenic rodents for experiments that require BL1 containment (See Appendix G-III-M, *Footnotes and References of Appendix G*) are exempt from the *NIH Guidelines*.

Appendix C-VIII. Generation of BL1 Transgenic Rodents via Breeding

The breeding of two different transgenic rodents or the breeding of a transgenic rodent and a non-transgenic rodent with the intent of creating a new strain of transgenic rodent that can be housed at BL1 containment will be exempt from the *NIH Guidelines* if:
(1) Both parental rodents can be housed under BL1 containment; and
(2) neither parental transgenic rodent contains the following genetic modifications: (i) incorporation of more than one-half of the genome of an exogenous eukaryotic virus from a single family of viruses; or (ii) incorporation of a transgene that is under the control of a gammaretroviral long terminal repeat (LTR); and
(3) the transgenic rodent that results from this breeding is not expected to contain more than one-half of an exogenous viral genome from a single family of viruses.

Appendix C-IX. Footnotes and References of Appendix C

Appendix C-IX-A. The NIH Director, with advice of the RAC, may revise the classification for the purposes of these *NIH Guidelines* (see Section IV-C-1-b-(2)-(b), *Minor Actions*). The revised list of organisms in each Risk Group is located in Appendix B.

Appendix C-IX-B. A subset of non-conjugative plasmid vectors are poorly mobilizable (e.g., pBR322, pBR313). Where practical, these vectors should be employed.

Appendix C-IX-C. Defined as observable under optimal laboratory conditions by transformation, transduction, phage infection, and/or conjugation with transfer of phage, plasmid, and/or chromosomal genetic information. Note that this definition of exchange may be less stringent than that applied to exempt organisms under Section

III-F-6, *Exempt Experiments.*

Appendix C-IX-D. As classified in the *Third Report of the International Committee on Taxonomy of Viruses: Classification and Nomenclature of Viruses*, R. E. F. Matthews (ed.), Intervirology 12 (129-296), 1979.

Appendix C-IX-E. i.e., the total of all genomes within a Family shall not exceed one-half of the genome.

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APPENDIX D. MAJOR ACTIONS TAKEN UNDER THE *NIH GUIDELINES*

As noted in the subsections of Section IV-C-1-b-(1), the Director, NIH, may take certain actions with regard to the *NIH Guidelines* after the issues have been considered by the RAC. Some of the actions taken to date include the following:

Appendix D-1. Permission is granted to clone foot and mouth disease virus in the EK1 host-vector system consisting of *E. coli* K-12 and the vector pBR322, all work to be done at the Plum Island Animal Disease Center.

Appendix D-2. Certain specified clones derived from segments of the foot and mouth disease virus may be transferred from Plum Island Animal Disease Center to the facilities of Genentech, Inc., of South San Francisco, California. Further development of the clones at Genentech, Inc., has been approved under BL1 + EK1 conditions.

Appendix D-3. The Rd strain of *Hemophilus influenzae* can be used as a host for the propagation of the cloned Tn 10 tet R gene derived from *E. coli* K-12 employing the non-conjugative *Hemophilus* plasmid, pRSF0885, under BL1 conditions.

Appendix D-4. Permission is granted to clone certain subgenomic segments of foot and mouth disease virus in HV1 *Bacillus subtilis* and *Saccharomyces cerevisiae* host-vector systems under BL1 conditions at Genentech, Inc., South San Francisco, California.

Appendix D-5. Permission is granted to Dr. Ronald Davis of Stanford University to field test corn plants modified by recombinant DNA techniques under specified containment conditions.

Appendix D-6. Permission is granted to clone in *E. coli* K-12 under BL1 physical containment conditions subgenomic segments of rift valley fever virus subject to conditions which have been set forth by the RAC.

Appendix D-7. Attenuated laboratory strains of *Salmonella typhimurium* may be used under BL1 physical containment conditions to screen for the *Saccharomyces cerevisiae* pseudouridine synthetase gene. The plasmid YEp13 will be employed as the vector.

Appendix D-8. Permission is granted to transfer certain clones of subgenomic segments of foot and mouth disease virus from Plum Island Animal Disease Center to the laboratories of Molecular Genetics, Inc., Minnetonka, Minnesota, and to work with these clones under BL1 containment conditions. Approval is contingent upon review of data on infectivity testing of the clones by a working group of the RAC.

Appendix D-9. Permission is granted to Dr. John Sanford of Cornell University to field test tomato and tobacco plants transformed with bacterial (*E.coli* K-12) and yeast DNA using pollen as a vector.

Appendix D-10. Permission is granted to Drs. Steven Lindow and Nickolas Panopoulos of the University of California, Berkeley, to release under specified conditions *Pseudomonas syringae*, pathovars (pv.) *syringae,* and *Erwinia herbicola* carrying *in vitro* generated deletions of all or part of the genes involved in ice nucleation.

Appendix D-11. Agracetus of Middleton, Wisconsin, may field test under specified conditions disease resistant tobacco plants prepared by recombinant DNA techniques.

Appendix D-12. Eli Lilly and Company of Indianapolis, Indiana, may conduct large-scale experiments and production involving *Cephalosporium acremonium* strain LU4-79-6 under less than Biosafety Level 1 - Large Scale (BL1-LS) conditions.

Appendix D-13. Drs. W. French Anderson, R. Michael Blaese, and Steven Rosenberg of the NIH, Bethesda, Maryland, can conduct experiments in which a bacterial gene coding for neomycin phosphotransferase will be inserted into a portion of the tumor infiltrating lymphocytes (TIL) of cancer patients using a retroviral vector, N2. The marked TIL then will be combined with unmarked TIL, and reinfused into the patients. This experiment is an addition to an ongoing adoptive immunotherapy protocol in which TIL are isolated from a patient's tumor, grown in culture in the presence of interleukin-2, and reinfused into the patient. The marker gene will be used to detect TIL at various time intervals following reinfusion.
Approval is based on the following four stipulations: (I) there will be no limitation of the number of patients in the continuing trial; (ii) the patients selected will have a life expectancy of about 90 days; (iii) the patients give fully informed consent to participate in the trial; and (iv) the investigators will provide additional data before inserting a gene for therapeutic purposes. (Protocol #8810-001)

Appendix D-14. U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) may conduct certain experiments involving products of a yellow fever virus originating from the 17-D yellow fever clone at the Biosafety Level 3 containment level using HEPA filters and vaccination of laboratory personnel.

In addition, USAMRIID may conduct certain experiments involving vaccine studies of Venezuelan equine encephalitis virus at the Biosafety Level 3 containment level using HEPA filters and vaccination of laboratory personnel.

Appendix D-15. Drs. R. Michael Blaese and W. French Anderson of the NIH, Bethesda, Maryland, can conduct experiments in which a gene coding for adenosine deaminase (ADA) will be inserted into T lymphocytes of patients with severe combined immunodeficiency disease, using a retroviral vector, LASN. Following insertion of the gene, these T lymphocytes will be reinfused into the patients. The patients will then be followed for evidence of clinical improvement in the disease state, and measurement of multiple parameters of immune function by laboratory testing.

Approval is based on the following two stipulations: (I) that intraperitoneal administration of transduced T lymphocytes not be used before clearance by the Chair of the Recombinant DNA Advisory Committee; and (ii) that the number of research patients be limited to 10 at this time.

In addition to the conditions outlined in the initial approval, patients may be given a supplement of a CD-34+-enriched peripheral blood lymphocytes (PBL) which have been placed in culture conditions that favor progenitor cell growth. This enriched population of cells will be transduced with the retroviral vector, G1NaSvAd. G1NaSvAd is similar to LASN, yet distinguishable by PCR. LASN has been used to transduce peripheral blood T lymphocytes with the ADA gene. Lymphocytes and myeloid cells will be isolated from patients over time and assayed for the presence of the LASN or G1NaSvAd vectors. The primary objectives of this protocol are to transduce CD 34+ peripheral blood cells with the adenosine deaminase gene, administer these cells to patients, and determine if such cells can differentiate into lymphoid and myeloid cells *in vivo*. There is a potential for benefit to the patients in that these hematopoietic progenitor cells may survive longer, and divide to yield a broader range of gene-corrected cells. (Protocol #9007-002)

Appendix D-16. Dr. Steven A. Rosenberg of the National Institutes of Health, Bethesda, Maryland, can conduct experiments on patients with advanced melanoma who have failed all effective therapy. These patients will be treated with escalating doses of autologous tumor infiltrating lymphocytes (TIL) transduced with a gene coding for tumor necrosis factor (TNF). Escalating numbers of transduced TIL will be administered at three weekly intervals along with the administration of interleukin-2 (IL-2). The objective is to evaluate the toxicity and possible therapeutic efficacy of the administration of tumor infiltrating lymphocytes (TIL) transduced with the gene coding for TNF. (Protocol #9007-003)

Appendix D-17. Dr. Malcolm K. Brenner of St. Jude Children's Research Hospital of Memphis, Tennessee, can conduct experiments on patients with acute myelogenous leukemia (AML). Using the LNL6 retroviral vector, the autologous bone marrow cells will be transduced with the gene coding for neomycin resistance. The purpose of this gene marking experiment is to determine whether the source of relapse after autologous bone marrow transplantation for acute myelogenous leukemia is residual malignant cells in the harvested marrow or reoccurrence of tumor in the patient. Determining the source of relapse should indicate whether or not purging of the bone marrow is a necessary procedure. (Protocol #9102-004)

Appendix D-18. Dr. Malcolm K. Brenner of St. Jude Children's Research Hospital of Memphis, Tennessee, can conduct experiments on pediatric patients with Stage D (disseminated) neuroblastoma who are being treated

with high-dose carboplatin and etoposide in either phase I/II or phase II trials. All the patients in these studies will be subjected to bone marrow transplantation since it will allow them to be exposed to chemoradiation that would be lethal were it not for the availability of stored autologous marrow for rescue. The bone marrow cells of these patients will be transduced with the gene coding for neomycin resistance using the LNL6 vector. The purpose of this gene marking study is to determine whether the source of relapse after autologous bone marrow transplantation is residual malignant cells in the harvested marrow or residual disease in the patient. Secondly, it is hoped to determine the contribution of marrow autographs to autologous reconstitution. (Protocol #9105-005/9105-006)

Appendix D-19. Dr. Albert B. Deisseroth of the MD Anderson Cancer Center of Houston, Texas, can conduct experiments on patients with chronic myelogenous leukemia who have been reinduced into a second chronic phase or blast cells. The patients in these studies will receive autologous bone marrow transplantation. Using the LNL6 vector, the bone marrow cells will be transduced with the gene coding for neomycin resistance. The purpose of these marking studies is to determine if the origin of relapse arises from residual leukemic cells in the patients or from viable leukemic cells remaining in the bone marrow used for autologous transplantation. (Protocol #9105-007)

Appendix D-20. Drs. Fred D. Ledley and Savio L. C. Woo of Baylor College of Medicine of Houston, Texas, can conduct experiments on pediatric patients with acute hepatic failure who are identified as candidates for hepatocellular transplantation. Using the LNL6 vector, the hepatocytes will be transduced with the gene coding for neomycin resistance. The purpose of using a genetic marker is to demonstrate the pattern of engraftment of transplanted hepatocytes and to help determine the success or failure of engraftment. (Protocol #9105-008)

Appendix D-21. Dr. Steven A. Rosenberg of the National Institutes of Health, Bethesda, Maryland, can conduct experiments on patients with advanced melanoma, renal cell cancer, and colon carcinoma who have failed all effective therapy. In an attempt to increase these patients' immune responses to the tumor, the tumor necrosis factor gene or the interleukin-2 gene will be introduced into a tumor cell line established from the patient. These gene-modified autologous tumor cells will then be injected into the thigh of the patient. To further utilize the immune system of the patient to fight the tumor, stimulated lymphocytes will be cultured from either the draining regional lymph nodes or the injected tumor itself. The patients will be evaluated for antitumor effects engendered by the injection of the gene modified tumor cells themselves as well as after the infusion of the cultured lymphocytes. (Protocol #9110-010/9110-011)

Appendix D-22. Dr. James M. Wilson of the University of Michigan Medical Center of Ann Arbor, Michigan, can conduct experiments on three patients with the homozygous form of familial hypercholesterolemia. Both children and adults will be eligible for this therapy. In an attempt to correct the basic genetic defect in this disease, the gene coding for the low-density lipoprotein (LDL) receptor will be introduced into liver cells taken from the patient. The gene-corrected hepatocytes will then be infused into the portal circulation of the patient through an indwelling catheter. The patients will be evaluated for engraftment of the these treated hepatocytes through a series of metabolic studies; three months after gene therapy, a liver biopsy will be taken and analyzed for the presence of recombinant derived RNA and DNA to document the presence of the gene coding for the normal LDL receptor. (Protocol #9110-012)

Appendix D-23. Dr. Michael T. Lotze of the University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, can conduct experiments on 20 patients with metastatic melanoma who have failed conventional therapy. A gene transfer experiment will be performed, transducing the patients' tumor infiltrating lymphocytes (TILs) with the gene for neomycin resistance. Through the use of this gene marking technique, it is proposed to determine how long TIL cells can be detected *in vivo* in the peripheral blood of the patients, and how the administration of interleukin-2 and interleukin-4 affects localization and survival of TIL cells in tumor sites. (Protocol #9105-009)

Appendix D-24. Dr. Gary J. Nabel of the University of Michigan Medical School, Ann Arbor, Michigan, can conduct gene therapy experiments on twelve patients with melanoma or adenocarcinoma. Patient population will be limited to adults over the age of 18 and female patients must be postmenopausal or have undergone tubal ligation or orchiectomy. The patient's immune response will be stimulated by the introduction of a gene encoding for a Class I MHC protein, HLA-B7, in order to enhance tumor regression. DNA/liposome-mediated transfection techniques will be used to directly transfer this foreign gene into tumor cells. HLA-B7 expression will be confirmed *in vivo*, and the immune response stimulated by the expression of this antigen will be characterized. These experiments will be analyzed for their efficacy in treating cancer. (Protocol #9202-013)

Appendix D-25. Kenneth Cornetta of Indiana University, Indianapolis, Indiana, can conduct gene transfer experiments on up to 10 patients with acute myelogenous leukemia (AML) and up to 10 patients with acute lymphocytic leukemia (ALL). The patient population will be limited to persons between 18 and 65 years of age. Using the LNL-6 vector, autologous bone marrow cells will be marked with the neomycin resistance gene. Gene marked and untreated bone marrow cells will be reinfused at the time of bone marrow transplantation. Patients

will then be monitored for evidence of the neomycin resistance gene in peripheral blood and bone marrow cells in order to determine whether relapse of their disease is a result of residual malignant cells remaining in the harvested marrow or inadequate ablation of the tumor cells by chemotherapeutic agents. Determining the source of relapse may indicate whether or not purging of the bone marrow is a necessary procedure for these leukemia patients. Further studies will be performed in order to determine the percentage of leukemic cells that contain the LNL-6 vector and the clonality of the marked cells. (Protocol #9202-014)

Appendix D-26. Dr. James S. Economou of the University of California, Los Angeles, can conduct gene transfer experiments on 20 patients with metastatic melanoma and 20 patients with renal cell carcinoma. These patients will be treated with various combinations of tumor-infiltrating lymphocytes and peripheral blood leukocytes, including CD8 and CD4 subsets of both types of cells. These effector cell populations will be given in combination with interleukin-2 (IL-2) in the melanoma patients and IL-2 plus alpha interferon in the renal cell carcinoma patients. The effector cells will be transduced with the neomycin resistance gene using either the LNL6 or G1N retroviral vectors. This "genetic marking" of the tumor-infiltrating lymphocytes and peripheral blood lymphocytes is designed to answer questions about the trafficking of these cells, their localization to tumors, and their *in vivo* life span. (Protocol #9202-015)

Appendix D-27. Drs. Philip Greenberg and Stanley R. Riddell of the Fred Hutchinson Cancer Research Center, Seattle, Washington, may conduct gene transfer experiments on 15 human immunodeficiency virus (HIV) seropositive patients (18-45 years old) undergoing allogeneic bone marrow transplantation for non-Hodgkin's lymphoma and 15 HIV-seropositive patients (18-50 years old) who do not have acquired immunodeficiency syndrome (AIDS)-related lymphoma and who are not undergoing bone marrow transplantation to evaluate the safety and efficacy of HIV-specific cytotoxic T lymphocyte (CTL) therapy. CTL will be transduced with a retroviral vector (HyTK) encoding a gene that is a fusion product of the hygromycin phosphotransferase gene (HPH) and the herpes simplex virus thymidine kinase (HSV-TK) gene. This vector will deliver both a marker gene and an ablatable gene in these T cell clones in the event that patients develop side effects as a consequence of CTL therapy. Data will be correlated over time, looking at multiple parameters of HIV disease activity. The objectives of these studies include evaluating the safety and toxicity of CTL therapy, determining the duration of *in vivo* survival of HIV-specific CTL clones, and determining if ganciclovir therapy can eradicate genetically modified, adoptively transferred CTL cells. (Protocol #9202-017)

Appendix D-28. Dr. Malcolm Brenner of St. Jude Children's Research Hospital, Memphis, Tennessee, can conduct gene therapy experiments on twelve patients with relapsed/refractory neuroblastoma who have relapsed after receiving autologous bone marrow transplant. In an attempt to stimulate the patient's immune response, the gene coding for Interleukin-2 (IL-2) will be used to transduce tumor cells, and these gene-modified cells will be injected subcutaneously in a Phase 1 dose escalation trial. Patients will be evaluated for an anti-tumor response. (Protocol #9206-018)

Appendix D-29. Drs. Edward Oldfield, Kenneth Culver, Zvi Ram, and R. Michael Blaese of the National Institutes of Health, Bethesda, Maryland, can conduct gene therapy experiments on ten patients with primary malignant brain tumors and ten patients with lung cancer, breast cancer, malignant melanoma, or renal cell carcinoma who have brain metastases. The patient population will be limited to adults over the age of 18. Patients will be divided into two groups based on the surgical accessibility of their lesions. Both surgically accessible and surgically inaccessible lesions will receive intra-tumoral injections of the retroviral Herpes simplex thymidine kinase (HS-tk) vector-producer cell line, G1TkSvNa, using a guided stereotaxic approach. Surgically accessible lesions will be excised seven days after stereotaxic injection, and the tumor bed will be infiltrated with the HS-tk producer cells. The removed tumor will be evaluated for the efficiency of transduction. Ganciclovir (GCV) will be administered beginning on the fifth postoperative day. In the case of surgically inaccessible lesions, the patients will receive intravenous therapy with GCV seven days after receiving the intra-tumoral injections of the retroviral HS-tk vector-producer cells. (Protocol #9206-019)

Appendix D-30. Dr. Albert D. Deisseroth of MD Anderson Cancer Center, Houston, Texas, can conduct gene transfer experiments on ten patients who have developed blast crisis or accelerated phase chronic myelogenous leukemia (CML). The retroviral vectors G1N and LNL6 which code for neomycin resistance will be

used to transduce autologous peripheral blood and bone marrow cells that have been removed and stored at the time of cytogenetic remission or re-induction of chronic phase in Philadelphia chromosome positive CML patients. Following reinduction of the chronic phase of CML and preparative chemotherapy, patients will be infused with the transduced autologous cells.

This protocol is designed to determine the cause of relapse of CML. If polyclonal CML neomycin marked blastic cells appear at the time of relapse, their presence will indicate that relapse arises from the leukemic CML blast cells present in the autologous cells infused following chemotherapy. If residual systemic disease contributes to relapse, the neomycin resistance gene will not be detected in the CML leukemic blasts at the time of relapse.

This study will compare the relative contributions of the peripheral blood and bone marrow to generate hematopoietic recovery after bone marrow transplantation and evaluate purging and selection of peripheral blood or bone marrow as a source of stem cells for transplant. The percentage of neomycin resistant CML cells which are leukemic will be determined by PCR analysis and detection of bcr-abl mRNA. (Protocol #9206-020)

Appendix D-31. Dr. Cynthia Dunbar of the National Institutes of Health, Bethesda, Maryland, can conduct gene transfer experiments on up to 48 patients with multiple myeloma, breast cancer, or chronic myelogenous leukemia. The retroviral vectors G1N and LNL6 will be used to transfer the neomycin resistance marker gene into autologous bone marrow and peripheral blood stem cells in the presence of growth factors to examine hematopoietic reconstitution after bone marrow transplantation. The efficiency of transduction of both short and long term autologous bone marrow reconstituting cells will be examined.

Autologous bone marrow and CD34+ peripheral blood stem cells will be enriched prior to transduction. Myeloma and CML patients will receive both autologous bone marrow and peripheral blood stem cell transplantation. These separate populations will be marked with both the G1N and LNL6 retroviral vectors. If short and long term marking experiments are successful, important information may be obtained regarding the biology of autologous reconstitution, the feasibility of retroviral gene transfer into hematopoietic cells, and the contribution of viable tumor cells within the autograft to disease relapse. (Protocol #9206-023/9206-024/9206-025)

Appendix D-32. Dr. Bernd Gansbacher of the Memorial Sloan-Kettering Cancer Center, New York, New York, can conduct gene therapy experiments on twelve patients over 18 years of age with metastatic melanoma who are HLA-A2 positive and who have failed conventional therapy. This is a phase I study to examine whether allogeneic HLA-A2 matched melanoma cells expressing recombinant human Interleukin-2 (IL-2) can be injected subcutaneously and used to create a potent tumor specific immune response without producing toxicity. By allowing the tumor cells to present the MHC Class I molecule as well as the secreted IL-2, a clonal expansion of tumor specific effector cells is expected. These effector populations may access residual tumor at distant sites via the systemic circulation. (Protocol #9206-021)

Appendix D-33. Dr. Bernd Gansbacher of the Memorial Sloan-Kettering Cancer Center, New York, New York, can conduct gene therapy experiments on twelve patients over 18 years of age with renal cell carcinoma who are HLA-A2 positive and who have failed conventional therapy. This Phase I study will examine whether allogeneic HLA-A2 matched renal cell carcinoma cells expressing recombinant human Interleukin-2 (IL-2) can be injected subcutaneously and used to create a potent tumor specific immune response without producing toxicity. By allowing the tumor cells to present the MHC Class I molecule as well as the secreted IL-2, a clonal expansion of tumor specific effector cells is expected. These effector populations may access residual tumor at distant sites via the systemic circulation. (Protocol #9206-022)

Appendix D-34. Dr. Michael T. Lotze, University of Pittsburgh, Pittsburgh, Pennsylvania, can conduct experiments on twenty patients with metastatic, and/or unresectable, locally advanced melanoma, renal cell carcinoma, breast cancer, or colon cancer who have failed standard therapy. Patients will receive multiple subcutaneous injections of autologous tumor cells combined with an autologous fibroblast cell line that has been transduced *in vitro* with the gene coding for Interleukin-4 (IL-4) to augment the *in vivo* antitumor effect. Patients will be monitored for antitumor effect by PCR analysis and multiple biopsy of the injection site. (Protocol #9209-033)

Appendix D-35. Dr. Friedrich G. Schuening, Fred Hutchinson Cancer Research Center, Seattle, Washington, can conduct human gene transfer experiments on patients ≥ 18 years of age with breast cancer, Hodgkin's disease, or non-Hodgkin's lymphoma. A total of 10 patients per year will be enrolled in the studies over a period of four years. Patients will undergo autologous bone marrow transplantation with a selected population of

Interleukin-3 (IL-3) or granulocyte colony-stimulating factor (G-CSF) stimulated CD34(+) peripheral blood repopulating cells (PBRC) that have been transduced with the gene coding for neomycin resistance (neo^R) using the retroviral vector, LN. Patients will be continuously monitored for neo^R to determine the relative contribution

of autologous PBRCs to long-term hematopoietic reconstitution. Demonstration of long-term contribution of autologous PBRC to hematopoiesis will enable the use of PBRC alone for autologous transplants and suggest the use of PBRC as long-term carriers of therapeutically relevant genes. (Protocol #9209-027/9209-028)

Appendix D-36. Dr. Friedrich G. Schuening, Fred Hutchinson Cancer Research Center, Seattle, Washington, can conduct human gene transfer experiments on patients ≥ 18 years of age with breast cancer, Hodgkin's disease, or non-Hodgkin's lymphoma. A total of 5 patients per year will be enrolled in the study over a period of four years. Patients will undergo allogeneic bone marrow transplant with granulocyte colony-stimulating factor (G-CSF) stimulated CD34(+) PBRC harvested from an identical twin that have been transduced with neo^R using the retroviral vector, LN. Patients will be continuously monitored for neo^R to determine the relative contribution of G-CSF stimulated allogeneic PBRCs to long-term bone marrow engraftment. Demonstration of long-term contribution of allogeneic PBRC to hematopoiesis will enable the use of PBRC alone for allogeneic transplants and suggest the use of PBRC as long-term carriers of therapeutically relevant genes. (Protocol #9209-029)

Appendix D-37. Dr. Malcolm K. Brenner of St. Jude Children's Hospital, Memphis, Tennessee, and Dr. Bonnie J. Mills of Baxter Healthcare Corporation, Santa Ana, California, can conduct a multicenter uncontrolled human gene transfer experiment on 12 patients ≤ 21 years of age with Stage D Neuroblastoma in first or second marrow remission. Autologous bone marrow cells will be separated into two fractions, purged and unpurged. Each fraction will be transduced with the neo^R gene by either LNL6 or G1Na. Patients will be monitored by the polymerase chain reaction (PCR) for the presence of neo^R. The protocol is designed to evaluate the safety and efficacy of the Neuroblastoma Bone Marrow Purging System following high dose chemotherapy. (Protocol #9209-032)

Appendix D-38. Drs. Carolyn Keierleber and Ann Progulske-Fox of the University of Florida, Gainesville, Florida, can conduct experiments involving the introduction of a gene coding for tetracycline resistance into *Porphyromonas gingivalis* at a physical containment level of Biosafety Level-2 (BL-2).

Appendix D-39. Dr. Scott M. Freeman of Tulane University Medical Center, New Orleans, Louisiana, can conduct experiments on patients with epithelial ovarian carcinoma who have clinical evidence of recurrent, progressive, or residual disease who have no other therapy available to prolong survival. Patients will be injected intraperitoneally with the irradiated PA-1 ovarian carcinoma cell line which has been transduced with the herpes simplex thymidine kinase (HSV-TK) gene. The patients will then receive ganciclovir therapy. Previous, data indicates that HSV-TK+ tumor cells exhibit a killing effect on HSV-TK- cells when exposed to ganciclovir therapy. Patients will be evaluated for safety and side effects of this treatment. (Protocol #9206-016)

Appendix D-40. Dr. Michael J. Welsh, Howard Hughes Medical Institute Research Laboratories, University of Iowa College of Medicine, Iowa City, Iowa, may conduct experiments on 3 cystic fibrosis (CF) patients ≥ 18 years of age with mild to moderate disease. This Phase I study will determine the: (1) *in vivo* safety and efficacy of the administration of the replication-deficient type 2 adenovirus vector, Ad2/CFTR-1, to the nasal epithelium; (2) efficacy in correcting the CF chloride transport defect *in vivo*; and (3) effect of adenovirus vector dosage on safety and efficacy. (Protocol #9212-036)

Appendix D-41. Dr. Ronald G. Crystal, National Institutes of Health, Bethesda, Maryland, may conduct experiments on 10 cystic fibrosis (CF) patients ≥ 21 years of age. Patients will receive an initial administration of the replication-deficient type 5 adenovirus vector, AdCFTR, to their left nares. If no toxicity is observed from intranasal administration, patients will receive a single administration of AdCFTR to the respiratory epithelium of their left large bronchi. Five groups of patients (2 patients per group) will be studied based on increased dosage administration of AdCFTR. This study will determine the: (1) *in vivo* safety and efficacy of the administration of AdCFTR into the respiratory epithelium; (2) efficacy of the correction of the biologic abnormalities of CF in the respiratory epithelium; (3) duration of the biologic correction; (4) efficacy of the correction of the abnormal electrical potential difference of the airway epithelial sheet; (5) clinical parameters relevant to the disease process; and (6) if humoral immunity develops against AdCFTR sufficient to prevent repeat administration. (Protocol #9212-034)

Appendix D-42. Dr. Kenneth Culver, Iowa Methodist Medical Center, Des Moines, Iowa, and Dr. John Van Gilder, University of Iowa, Iowa City, Iowa, may conduct experiments on 15 patients ≥ 18 years of age with

recurrent malignant primary brain tumors or lung, melanoma, renal cell carcinoma, or breast carcinoma brain metastases who have failed standard therapy for their disease. Patient eligibility will be limited to those patients

who have measurable residual tumor immediately following the post-operative procedure as demonstrated by imaging studies. The number of patients treated will be equally divided between the Iowa Methodist Medical Center and the University of Iowa. If a positive response is observed in any of the first 15 patients, the investigators may submit a request to treat an additional 15 patients.
Following surgical debulking, patients will receive a maximum of 3 intralesional injections of the G1TkSvNa vector- producing cell line (VPC) to induce regression of residual tumor cells by ganciclovir (GCV) therapy. Patients who demonstrate stable disease for a minimum of 6 months following this treatment will be eligible for additional VPC injections and subsequent GCV therapy. (Protocol #9303-037)

Appendix D-43. Drs. Malcolm Brenner, Robert Krance, Helen E. Heslop, Victor Santana, and James Ihle, St. Jude Children's Research Hospital, Memphis, Tennessee, may conduct experiments on 35 patients ≥ 1 year and ≤ 21 years of age at the time of initial diagnosis of acute myelogenous leukemia (AML). The investigators will use the two retroviral vectors, LNL6 and G1Na, to determine the efficacy of the bone marrow purging techniques: 4-hydroxyperoxicyclophosphamide and interleukin-2 (IL-2) activation of endogenous cytotoxic effector cells, in preventing relapse from the reinfusion of autologous bone marrow cells. (Protocol #9303-039)

Appendix D-44. Drs. Helen E. Heslop, Malcolm Brenner, and Cliona Rooney, St Jude Children's Research Hospital, Memphis, Tennessee, may conduct experiments of 35 patients ≤ 21 years of age who will be recipients of mismatched-related or phenotypically similar unrelated donor marrow grafts for leukemia. In this Phase I dose escalation study, spontaneous lymphoblastoid cell lines will be established that express the same range of Epstein-Barr Virus (EBV) encoded proteins as the recipient. These EBV-specific cell lines will be transduced with LNL6 or G1Na and readministered at the time of bone marrow transplant. This study will determine: (1) survival and expansion of these EBV-specific cell lines *in vivo*, (2) the ability of these adoptively transferred cells to confer protection against EBV infection, and (3) appropriate dosage and administration schedules. (Protocol #9303-038)

Appendix D-45. Drs. Robert W. Wilmott and Jeffrey Whitsett, Children's Hospital Medical Center, Cincinnati, Ohio, and Dr. Bruce Trapnell, Genetic Therapy, Inc., Gaithersburg, Maryland, may conduct experiments on 15 cystic fibrosis (CF) patients who have mild to moderate disease ≥ 21 years of age. The replication-deficient type 5 adenovirus vector, Av1CF2, will be administered to the nasal and lobar bronchial respiratory tract of patients. This study will demonstrate the: (1) expression of normal cystic fibrosis transmembrane conductance regulator (CFTR) mRNA *in vivo*, (2) synthesis of CFTR protein, and (3) correction of epithelial cell cAMP dependent Cl^- permeability. The pharmacokinetics of CFTR expression and ability to re-infect the respiratory tract with AvCF2 will be determined. Systemic and local immunologic consequences of Av1CF2 infection, the time of viral survival, and potential for recombination or complementation of the virus will be monitored. (Protocol #9303-041)

Appendix D-46. Dr. James M. Wilson of the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania, may conduct experiments on 20 adult patients with advanced cystic fibrosis lung disease. An isolated segment of the patients' lung will be transduced with the E1 deleted, replication-incompetent adenovirus vector, Ad.CB-CFTR using a bronchoscope for gene delivery. Ad.CB-CFTR contains the human gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Pulmonary biopsies will be obtained by bronchoscopy at 4 days, 6 weeks, and 3 months following treatment. Patients will be monitored for evidence of CFTR gene transfer and expression, immunological responses to CFTR or adenovirus proteins, and toxicity. (Protocol #9212-035)

Appendix D-47. Dr. Hilliard F. Seigler of Duke University Medical Center, Durham, North Carolina, may conduct experiments on 20 patients with disseminated malignant melanoma. Autologous tumor cells will be transduced with a retroviral vector, pHuγ-IFN, that contains the gene encoding human γ-IFN. Following lethal irradiation, the transduced cells will be readministered to patients for the purpose of generating cytotoxic T cells that are tumor specific along with the up-regulation of Class I major histocompatibility antigens. Patients will be monitored for clinical regression of tumors and generation of tumor-specific cytotoxic T lymphocytes. (Protocol #9306-043)

Appendix D-48. Drs. Stefan Karlsson and Cynthia Dunbar of the National Institutes of Health, Bethesda, Maryland, and Dr. Donald B. Kohn of the Children's Hospital of Los Angeles, Los Angeles, California, may conduct experiments on 10 patients with Gaucher disease. CD34(+) hematopoietic stem cells will be isolated

from bone marrow or from peripheral blood treated with granulocyte-colony stimulating factor. CD34(+) cells will be transduced with a retrovirus vector, G1Gc, containing cDNA encoding human glucocerebrosidase and administered intravenously. Patients will be monitored for toxicity and glucocerebrosidase expression. (Protocol #9306-047)

Appendix D-49. Dr. Gary J. Nabel of the University of Michigan Medical Center, Ann Arbor, Michigan, may conduct experiments on 12 patients with AIDS to be divided into 4 experimental groups. CD4(+) lymphocytes will be isolated from peripheral blood and transduced with Rev M10, a transdominant inhibitory mutant of the *rev* gene of the human immunodeficiency virus (HIV). Transduction of the *rev* mutant will be mediated either by the retrovirus vector, PLJ-cREV M10, or by particle-mediated gene transfer of plasmid DNA. Patients will be monitored for survival of the transduced CD4(+) cells by polymerase chain reaction and whether Rev M10 can confer protection against HIV infection to CD4(+) cells. (Protocol #9306-049)

Appendix D-50. Dr. Gary J. Nabel of the University of Michigan Medical Center, Ann Arbor, Michigan, may conduct experiments on 24 patients with advanced cancer. Patients will undergo *in vivo* transduction with DNA/liposome complexes containing genes encoding the HLA-B7 histocompatibility antigen and beta-2 microglobulin in a non-viral plasmid. These DNA/liposome complexes will be administered either by intratumoral injection or catheter delivery. Patients will be monitored for enhanced immune responses against tumor cells, and safe and effective doses will be determined. (Protocol #9306-045)

Appendix D-51. Dr. John A. Barranger of the University of Pittsburgh, Pittsburgh, Pennsylvania, may conduct experiments on 5 patients with Gaucher disease. The CD34(+) hematopoietic stem cells will be isolated from peripheral blood and transduced *in vitro* with the retrovirus vector, N2-Sv-GC, encoding the glucocerebrosidase (GC) enzyme. Following reinfusion of the transduced cells, patients will be monitored by PCR analysis for GC expression in peripheral blood leukocytes. Patients currently receiving GC replacement therapy and who demonstrate clinical responsiveness will be withdrawn from exogenous GC therapy. Patients not previously treated with exogenous GC, will be monitored for clinical reversal of lipid storage symptoms. (Protocol #9306-046)

Appendix D-52. Dr. Robert Walker of the National Institutes of Health, Bethesda, Maryland, may conduct experiments on 12 HIV-infected patients who have a seronegative identical twin. CD4(+) and CD8(+) cells will be isolated from the seronegative twin and induced to polyclonal proliferation with anti-CD3 and interleukin-2. The enriched population of cells will be transduced with either LNL6 or G1Na, which contain the neo^R gene. The transduced cells will be expanded in tissue culture and administered to the HIV-infected twin. Patients will be monitored for immune function and the presence of marked cells. (Protocol #9209-026)

Appendix D-53. Dr. Corey Raffel of the Children's Hospital Los Angeles, Los Angeles, California, and Dr. Kenneth Culver of Iowa Methodist Medical Center, Des Moines, Iowa, may conduct experiments on 30 patients between 2 and 18 years of age with recurrent malignant astrocytoma. Fifteen patients will be accrued into this study initially. If at least one patient responds to therapy, an additional 14 patients will be treated. Patients with either surgically accessible or non-accessible tumors will be treated with the vector producing cell line (PA317) carrying the retrovirus vector, G1TkSvNa. This vector will transduce tumor cells *in vivo* with the *Herpes simplex* thymidine kinase (HS-tk) gene that renders the cells sensitive to killing by ganciclovir. Surgically accessible patients will undergo surgical debulking of their tumor followed by repeated administration of the HS-tk vector producer cells into the tumor bed. Children with unresectable tumors will undergo stereotaxic injection of vector producer cells into tumors. (Protocol #9306-050)

Appendix D-54. Dr. Jeffrey E. Galpin of the University of Southern California, Los Angeles, California, and Dr. Dennis A. Casciato of the University of California, Los Angeles, California, may conduct experiments on 15 HIV(+) asymptomatic patients. Patients will receive 3 monthly intramuscular injections of the retrovirus vector (N2IIIBenv) encoding the HIV-1 IIIB envelope protein. Patients will be monitored for acute toxicity, CD4 levels, HIV-specific CTL responses, and viral burdens. (Protocol #9306-048)

Appendix D-55. Drs. Charles Hesdorffer and Karen Antman of Columbia University College of Physicians and Surgeons, New York, New York, may conduct experiments on 20 patients with advanced breast, ovary, and brain cancer. CD34(+) hematopoietic stem cells will be isolated from bone marrow, transduced with the retrovirus vector, PHaMDR1/A, and readministered to patients. Patients will be monitored for the presence and expression of the MDR-1 gene. The investigators will determine whether MDR-1 expression increases following chemotherapy. (Protocol #9306-051)

Appendix D-56. Dr. Enzo Paoletti of Virogenetics Corporation, Troy, New York, may conduct experiments with poxvirus vectors NYVAC, ALVAC, and TROVAC at Biosafety Level 1.

Appendix D-57. Drs. Richard C. Boucher and Michael R. Knowles of the University of North Carolina, Chapel Hill, North Carolina, may conduct experiments on 9 patients (18 years old or greater) with cystic fibrosis to test for the safety and efficacy of an E1-deleted recombinant adenovirus containing the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA, Ad.CB-CFTR. A single dose of 10^8, 3 x 10^9 or 10^{11} pfu/ml will be administered to the nasal cavity of 3 patients in each dose group. Patients will be monitored by nasal lavage and biopsy to assess safety and restoration of normal epithelial function. (Protocol #9303-042)

Appendix D-58. Dr. Joyce A. O'Shaughnessy of the National Institutes of Health, Bethesda, Maryland, may conduct experiments on 18 patients (18-60 years old) with Stage IV breast cancer who have achieved a partial or complete response to induction chemotherapy. This study will determine the feasibility of obtaining engraftment of CD34(+) hematopoietic stem cells transduced by a retroviral vector, G1MD, and expressing a cDNA for the human multi-drug resistance-1 (MDR-1) gene following high dose chemotherapy, and whether the transduced MDR-1 gene confers drug resistance to hematopoietic cells and functions as an *in vivo* dominant selectable marker. Patients will be monitored for evidence of myeloprotection and presence of the transduced MDR-1 gene." (Protocol #9309-054)

Appendix D-59. Drs. Larry E. Kun, R. A. Sanford, Malcolm Brenner, and Richard L. Heideman of St. Jude Children's Research Hospital, Memphis, Tennessee, and Dr. Edward H. Oldfield of the National Institutes of Health, Bethesda, Maryland, may conduct experiments on 6 patients (3-21 years old) with progressive or recurrent malignant supratentorial tumors resistant to standard therapies. Mouse cells producing the retroviral vector containing the herpes simplex thymidine kinase gene (G1TKSVNa) will be instilled into the tumor areas via multiple stereotactically placed cannulas. Patients will be treated with ganciclovir to eliminate cells expressing the transduced gene. Patients will be monitored for central nervous system, hematologic, renal or other toxicities, and for anti-tumor responses by magnetic resonance imaging studies. (Protocol #9309-055)

Appendix D-60. The physical containment level may be reduced from Biosafety Level 3 to Biosafety Level 2 for a Semliki Forest Virus (SFV) vector expression system of Life Technologies, Inc., Gaithersburg, Maryland.

Appendix D-61. Dr. Albert B. Deisseroth of the University of Texas MD Anderson Cancer Center, Houston, Texas, may conduct experiments on 10 patients (≥ 16 to ≤ 60 years of age) with chronic lymphocytic leukemia. Autologous peripheral blood and bone marrow cells will be removed from patients following chemotherapy and marked by transduction with two distinguishable retroviral vectors, G1Na and LNL6, containing the neomycin resistance gene. The gene marked cells will be reinfused into patients to determine the efficiency of bone marrow purging and the origin of relapse following autologous bone marrow transplantation. (Protocol #9209-030)

Appendix D-62. Dr. Jonathan Simons of the Johns Hopkins Oncology Center, Baltimore, Maryland, may conduct experiments on 26 patients (≥ 18 years of age) with metastatic renal cell carcinoma to evaluate the safety and tolerability of intradermally injected autologous irradiated tumor cells transduced with the retrovirus vector, MFG, containing the human granulocyte-macrophage colony stimulating factor gene. Acute and long-term clinical toxicities and *in vitro* and *in vivo* induction of specific anti-tumor immune responses will be monitored. (Protocol #9303-040)

Appendix D-63. Dr. Albert B. Deisseroth of the University of Texas MD Anderson Cancer Center, Houston, Texas, may conduct experiments on 20 patients (≥ 18 and ≤ 60 years old) with ovarian cancer. A murine viral vector was constructed from the third generation of L series retroviruses with the insert of the human multi-drug resistance-1 (MDR-1) transduced gene. The investigators will assess the safety and feasibility of administering CD34 (+) autologous peripheral blood and bone marrow cells. Patients will be monitored for the presence of the MDR-1 gene and for the effect of gene transfer on hematopoietic function following the transplantation. (Protocol #9306-044)

Appendix D-64. Dr. Joseph Ilan of the Case Western Reserve University School of Medicine and University Hospital of Cleveland, Cleveland, Ohio, may conduct experiments on 12 patients (≥ 18 years of age) with advanced brain cancer. Human malignant glioma tumor cells will be cultured, transfected with Epstein-Barr virus (EBV)-based vector, anti-Insulin growth factor-I, lethally irradiated, and injected subcutaneously into

patients in an attempt to express antisense Insulin growth factor-1. Patients will be monitored for toxicity and immunologic responses to the vector. (Protocol #9306-052)

Appendix D-65. Drs. James S. Economou and John Glaspy of the University of California, Los Angeles, California, may conduct experiments on 30 patients (≥ 18 to ≤ 70 years of age) with metastatic melanoma. A human melanoma cell line (M-24) will be transduced with the retroviral vector, G1NaCvi2, expressing the human interleukin-2 (IL-2) gene. The IL-2 producing cells will be mixed with the patient's autologous tumor cells, irradiated, and injected subcutaneously in an attempt to enhance the tumor-specific immunologic response. Patients will be monitored for toxicity, *in vitro* and *in vivo* immunologic responses, and clinical anti-tumor effects. (Protocol #9309-058)

Appendix D-66. Drs. Peter Cassileth, Eckhard R. Podack, and Kasi Sridhar of the University of Miami, and Niramol Savaraj of the Miami Veterans Administration Hospital, Miami, Florida, may conduct experiments on 12 patients (≥ 18 years of age) with limited stage small cell lung cancer. Autologous tumor cells will be removed, expanded in culture, and transduced by lipofection with the BMG-Neo-hIL2 vector (derived from bovine papilloma virus). The objective of this protocol is to demonstrate the safety and efficacy of administering IL-2 transduced autologous tumor cells in an attempt to stimulate a tumor-specific cytotoxic T lymphocyte (CTL) response, and to determine the quantity and characteristics of the CTL that have been generated. (Protocol #9309-053)

Appendix D-67. Drs. Edward H. Oldfield and Zvi Ram of the National Institutes of Health, Bethesda, Maryland, may conduct experiments on 20 patients (≥ 18 years of age) with leptomeningeal carcinomatosis. The patients will receive intraventricular or subarachnoid injection of murine vector producing cells containing the retroviral vector, G1Tk1SvNa. Tumor cells expressing the herpes simplex thymidine kinase gene will be rendered sensitive to killing by subsequent administration of ganciclovir. Patients will be monitored for safety and anti-tumor response by magnetic resonance imaging (MRI) and cerebral spinal fluid cytological analysis. (Protocol #9312-059)

Appendix D-68. Drs. Tapas K. Das Gupta and Edward P. Cohen of the University of Illinois College of Medicine, Chicago, Illinois, may conduct experiments on 12 subjects who differ in at least 3 out of 6 alleles at the Class I histocompatibility locus (≥ 18 years of age) with Stage IV malignant melanoma. The subjects will be immunized with a lethally irradiated allogeneic human melanoma cell line transduced with the human interleukin-2 expressing retroviral vector, pZipNeoSv-IL-2. Subjects will be monitored for toxicity, induction of B and T cell antitumor responses *in vitro* and *in vivo,* and any clinical evidence of antitumor effect. (Protocol #9309-056)

Appendix D-69A. Dr. Michael J. Welsh of the Howard Hughes Medical Institute, Iowa City, Iowa, may conduct experiments on 20 patients (≥ 18 years of age) with cystic fibrosis. The investigator will administer increasing doses of either of the two adenovirus vectors, AD2/CFTR-1 or AD2-ORF6/PGK-CFTR, to the nasal epithelium of 10 patients (1 nostril) or maxillary sinus epithelium of 10 patients (1 maxillary sinus). Patients will be isolated for a period of 24 hours following vector administration; however, if 1 patient demonstrates secreted virus at 24 hours, the investigator will notify the Recombinant DNA Advisory Committee for reconsideration of the isolated period. Patients will be assessed for the safety and efficacy of multiply administration of adenovirus vectors encoding the cystic fibrosis transmembrane conductance regulator (CFTR) gene. (Protocol #9312-067)

Appendix D-69B. Dr. Richard Haubrich of the University of California at San Diego Treatment Center, San Diego, California, may conduct experiments on 25 human immunodeficiency virus (HIV)-infected, seropositive, asymptomatic subjects (≥ 18 to ≤ 65 years of age). Subjects will receive 3 monthly intramuscular injections of the retroviral vector, N2/IIIB *env/rev*, which encodes for HIV-1 IIIB *env/rev* proteins. The objective of the study is to induce HIV-1- specific CD8(+) cytotoxic T lymphocyte and antibody responses in order to eliminate HIV-infected cells and residual virus. This Phase I/II study will evaluate acute toxicity, identify long-term treatment effects, and evaluate the disease progression. (Protocol #9312-062)

Appendix D-70. Dr. Mario Sznol of the National Institutes of Health, Frederick, Maryland, may conduct experiments on 50 subjects (≥ 18 years of age) with advanced stage melanoma. Subjects will receive subcutaneous injections of lethally irradiated allogeneic melanoma cells that have been transduced by lipofection with the plasmid DNA vector, CMV-B7, derived from bovine papilloma virus to express the human B7 antigen. The B7 antigen, which binds to the CD28 receptor of T cells, will serve as a co-stimulatory signal to elicit an antitumor immune response. Subjects will be monitored for induction of cytotoxic T lymphocyte, antitumor responses *in vitro* and *in vivo* and any clinical evidence of antitumor effect. (Protocol #9312-063)

Appendix D-71. Dr. Joseph Rubin of the Mayo Clinic, Rochester, Minnesota, may conduct experiments on 15 subjects with hepatic metastases from advanced colorectal cancer (≥ 18 years of age). Subjects will receive intratumoral hepatic injections of the plasmid DNA/lipid complex, pHLA-B7/β-2 microglobulin, expressing a heterodimeric cell surface protein consisting of the HLA-B7 histocompatibility antigen and β-2 microglobulin. Subjects must be HLA-B7 negative. The objective of this study is to determine a safe and effective dose of the DNA/lipid complex. Subjects will be monitored for antigen-specific immune responses and *in vivo* HLA-B7 expression. (Protocol #9312-064)

Appendix D-72. Dr. Nicholas J. Vogelzang of the University of Chicago Medical Center, Chicago, Illinois, may conduct experiments on 15 subjects with metastatic renal cell carcinoma ≥ 18 years of age. Subjects will receive intratumoral injections of the plasmid DNA/liposome vector pHLA-B7/β-2 microglobulin, expressing a heterodimeric cell surface protein consisting of the HLA-B7 histocompatibility antigen and β-2 microglobulin. Subjects must be HLA-B7 negative. Subjects will be monitored for antigen-specific immune responses and *in vivo* HLA-B7 expression. (Protocol #9403-071)

Appendix D-73. Dr. Evan M. Hersh of the Arizona Cancer Center and Drs. Emmanuel Akporiaye, David Harris, Alison T. Stopeck, Evan C. Unger, and James A. Warneke of the University of Arizona, Tucson, Arizona, may conduct experiments on 15 subjects with metastatic malignant melanoma ≥ 18 years of age. Subjects will receive intratumoral injections of the plasmid DNA/liposome vector, pHLA-B7/β-2 microglobulin, expressing a heterodimeric cell surface protein consisting of the HLA-B7 histocompatibility antigen and β-2 microglobulin. Subjects must be HLA-B7 negative. Subjects will be monitored for antigen-specific immune responses and *in vivo* HLA-B7 expression. (Protocol #9403-072)

Appendix D-74. Dr. Ralph Freedman of MD Anderson Cancer Center, Houston, Texas, may conduct gene marking experiments on 9 subjects with ovarian carcinoma or peritoneal carcinomatosis (≥ 16 years of age). Autologous CD3(+)/CD8(+) tumor infiltrating lymphocyte derived T cells will be transduced with the retroviral vector G1Na that encodes for neo^R. Subjects will receive intraperitoneal administration of bulk expanded transduced and nontransduced T cells and recombinant interleukin-2. Previously documented tumor sites and normal tissues will be monitored for neo^R and the proportion of CD3(+)/CD8(+) T cells will be determined. (Protocol #9406-075)

Appendix D-75. Drs. Helen Heslop, Malcolm Brenner, and Robert Krance of St. Jude Children's Research Hospital, Memphis, Tennessee, may conduct gene marking experiments on 20 subjects undergoing autologous bone marrow transplantation for therapy of leukemia or solid tumor (< 21 years of age). The distinguishable retroviral vectors, LNL6 and G1Na (both encoding neo^R), will be used to determine the rate of reconstitution of untreated versus cytokine expanded CD34(+) selected autologous bone marrow cells. (Protocol #9406-076)

Appendix D-76. Drs. Albert Deisseroth, Gabriel Hortobagyi, Richard Champlin, and Frankie Holmes of MD Anderson Cancer Center, Houston, Texas, may conduct experiments on 10 fully evaluable subjects (maximum of 20 entered) with Stage III or IV breast cancer (≥ 18 and ≤ 60 years of age). Subjects will receive autologous CD34(+) peripheral blood cells that have been transduced with the retroviral vector, pVMDR-1, which encodes the multi-drug resistance gene. The objective of this study is to evaluate the safety and feasibility of transducing early hematopoietic progenitor cells with pVMDR-1 and to determine *in vivo* selection of chemotherapy resistant hematopoietic cells. (Protocol #9406-077)

Appendix D-77. Drs. Johnson M. Liu and Neal S. Young of the National Institutes of Health, Bethesda, Maryland, may conduct experiments on 6 patients with Fanconi anemia (≥ 5 years of age). Subjects will receive autologous CD34(+) cells that have been transduced with the retroviral vector, FACC, which encodes the normal Fanconi anemia complementation group C gene. The objective of this study is to determine whether autologous FACC transduced hematopoietic progenitor cells can be safely administered to subjects, the extent of engraftment, and correction of cell phenotype. (Protocol #9406-078)

Appendix D-78. Drs. Robert E. Sobol and Ivor Royston of the San Diego Regional Cancer Center, San Diego, California, may conduct experiments on 15 subjects with recurrent residual glioblastoma multi-forme (≥ 18 years of age). Subjects will receive subcutaneous injections of autologous tumor cells that have been lethally irradiated and transduced with the retroviral vector, G1NaCvi2.23, which encodes for interleukin-2. Subjects will be monitored *in vitro* for cellular and humoral antitumor responses and *in vivo* for antitumor activity. (Protocol #9406-080)

Appendix D-79. Dr. Alfred E. Chang of the University of Michigan Medical Center, Ann Arbor Michigan, may conduct gene marking experiments on 15 subjects with metastatic melanoma (≥ 18 years of age). Subjects will undergo adoptive immunotherapy of anti-CD3/interleukin-2 activated lymph node cells that have been primed *in vivo* with tumor cells that have been transduced with the retrovirus vector, GBAH4, encoding the gene for interleukin-4. The investigator will evaluate the antitumor efficacy and *in vivo* immunological reactivity in subjects receiving adoptively transferred T cells, and the *in vitro* immunological reactivities of the activated T cells that might correlate with their *in vivo* antitumor function. (Protocol #9312-065)

Appendix D-80. Dr. Robert Walker of the National Institutes of Health, Bethesda, Maryland, may conduct gene marking experiments on 40 HIV(+) subjects (≥ 18 years of age). The investigator may also enter an additional number of subjects (to be determined by the investigator) who will receive a single administration of 1×10^7 HIV-specific CD8(+) cells. The investigator will: (1) Assess the safety and tolerance of the adoptive transfer of anti-HIV cytotoxic, syngeneic, CD8(+) peripheral blood lymphocytes that have been transduced with the retrovirus vector, r*kat*4svgF3e-, that encodes for a universal chimeric T cell receptor. (2) Determine the longevity of the genetically marked CD8(+) lymphocytes in the subject's peripheral blood. (Protocol #9403-069)

Appendix D-81. Dr. Joseph Rosenblatt of the University of California, Los Angeles, California, and Dr. Robert Seeger of Children's Hospital, Los Angeles, California, may conduct gene transfer experiments on 18 subjects with neuroblastoma (≤ 21 years of age). Patients at high risk of relapse with minimal or no detectable disease following myeloablative therapy and autologous bone marrow transplantation, or patients with progressive or persistent disease despite conventional therapy will be serially immunized with autologous or allogeneic neuroblastoma cells transduced to express γ interferon. Cells will be transduced with the retroviral vector, pHuγ-IFN, encoding the human gene for γ interferon and lethally irradiated prior to use as an immunogen. The objectives of the study are: (1) to determine the maximum tolerable dose of transduced cells; (2) to determine the local, regional, and systemic toxicities of injected cells; and (3) to determine the antitumor response *in vivo* as measured by standard clinical tests and immunocytologic evaluation of marrow metastases. (Protocol #9403-068)

Appendix D-82. Dr. Kenneth L. Brigham of Vanderbilt University, Nashville, Tennessee, may conduct gene transfer experiments on 10 subjects (≤ 21 years of age) in two different patient protocols (5 for each protocol). Both protocols will use the same DNA/liposome preparations to deliver a plasmid DNA construct, pCMV4-AAT, encoding human alpha-1 antitrypsin gene driven by a cytomegalovirus promoter. In patients scheduled for elective pulmonary resection, the DNA/liposome complexes will be instilled through a fiber optic bronchoscope into a subsegment of the lung. Tissues of the lung will be obtained at the time of surgery. Transgene expression will be assessed by immunohistochemistry, *in situ* hybridization, and Western and Northern blot analyses. The effect of DNA/liposome complex administration on the histological appearance of the lung will also be evaluated. In patients with alpha-1 antitrypsin deficiency, the DNA/liposome complexes will be instilled into the nostril. Transgene expression will be determined in cells obtained by nasal lavage and nasal scraping, and the time course of transgene expression will be measured. The secretion of the alpha-1 antitrypsin protein in nasal fluid will be determined. Histological appearance of nasal mucosa will also be examined. The study will assess safety and feasibility of gene delivery to the human respiratory tract. (Protocol #9403-070)

Appendix D-83. Dr. H. Kim Lyerly of Duke University Medical Center, Durham, North Carolina, may conduct gene transfer experiments on 20 subjects with refractory or recurrent metastatic breast cancer (≥ 18 years of age). Autologous breast cancer cells will be transduced with the DNA/liposome complex, pMP6-IL2, containing a plasmid DNA vector derived from adeno-associated virus (AAV) that expresses the gene for human interleukin-2. Subjects will receive 4 subcutaneous injections of lethally irradiated tumor cells transduced with the DNA/liposome complex prior to injection. The objective of this study is to: (1) evaluate the safety and toxicity of the treatment, (2) determine the immunological effects, (3) determine the duration of clinical responses, and (4) measure patient survival. (Protocol #9409-086)

Appendix D-84. Drs. Flossie Wong-Staal, Eric Poeschla, and David Looney of the University of California at San Diego, La Jolla, California, may conduct gene transfer experiments on 6 subjects (≥ 18 and ≤ 65 years of age) infected with human immunodeficiency virus-1 (HIV-1). Autologous CD4(+) T lymphocytes will be transduced *ex vivo* with the retroviral vector, pMJT, expressing a hairpin ribozyme that cleaves the HIV-1 RNA in the 5' leader sequence. The transduced cells will be expanded and reinfused into the patients. The objectives of the study are: (1) to evaluate safety of reinfusing the transduced lymphocytes, (2) to compare (*in vivo*) the kinetics and survival of ribozyme-transduced cells with that of cells transduced with a control vector, (3) to determine *in vivo* expression of the ribozyme sequences in transduced lymphocytes, (4) to determine whether host immune responses directed against the transduced cells will occur *in vivo*, and (5) to obtain preliminary

data on the effects of ribozyme gene therapy on *in vivo* HIV mRNA expression, viral burden and CD4(+) lymphocyte levels. (Protocol #9309-057)

Appendix D-85. Dr. Friedrich Schuening of the Fred Hutchinson Cancer Research Center, Seattle, Washington, may conduct gene transfer experiments on 10 subjects (≥ 18 years of age) with Type I Gaucher's disease. The peripheral blood repopulating cells (mobilized by patient pretreatment with recombinant granulocyte colony-stimulating factor) will be harvested and CD34(+) cells selected. CD34(+) cells will be transduced *ex vivo* with the retroviral vector, LgGC, that encodes human glucocerebrosidase cDNA. Following transduction, the transduced cells will be infused into the patient without myeloablative treatment. The primary endpoint of this study is to examine the safety of infusing CD34(+) cells transduced with the human glucocerebrosidase cDNA. Patients will be monitored for persistence and expression of the glucocerebrosidase gene in hematopoietic cells. (Protocol #9312-061)

Appendix D-86. Dr. Terence R. Flotte of the Johns Hopkins Children's Center, Baltimore, Maryland, may conduct gene transfer experiments on 16 subjects (≥ 18 years of age) with mild cystic fibrosis (CF). An adeno-associated virus (AAV) derived vector, encoding cystic fibrosis transmembrane conductance regulator (CFTR) gene, (tgAAVCF), will be administered to nasal (direct) and airway (bronchoscope) epithelial cells. This is a dose escalation study involving 8 cohorts. Each subject will receive both intranasal and bronchial administration of the adenoviral vector at 4 escalating doses. Nasal doses will range between 1×10^6 and 1×10^9 pfu. Lung administration will range between 1×10^7 and 1×10^{10} pfu. The primary goal of the study is to assess the safety of vector administration. Respiratory and nasal epithelial cells will be evaluated for gene transfer, expression, and physiologic correction. (Protocol #9409-083)

Appendix D-87. Drs. Jeffrey M. Isner and Kenneth Walsh of St. Elizabeth's Medical Center, Tufts University, Boston, Massachusetts, may conduct gene transfer experiments on 12 subjects (≥ 40 years of age) with peripheral artery disease (PAD). A plasmid DNA vector, phVEGF165, encoding the human gene for vascular endothelial growth factor (VEGF) will be used to express VEGF to induce collateral neovascularization. Percutaneous arterial gene transfer will be achieved using an angioplasty catheter with a hydrogel coated balloon to deliver the plasmid DNA vector to the artery. The objectives of the study are: (1) to determine the efficacy of arterial gene therapy to relieve rest pain and/or heal ischemic ulcers of the lower extremities in patients with PAD; and (2) to document the safety of the phVEGF arterial gene therapy for therapeutic angiogenesis. Subjects will undergo anatomic and physiologic examination to determine the extent of collateral artery development following phVEGF arterial gene therapy. (Protocol #9409-088)

Appendix D-88A. Dr. Ronald G. Crystal of New York Hospital-Cornell Medical Center, New York, New York, may conduct gene transfer experiments on 26 patients (≥ 15 years of age) with cystic fibrosis (CF). A replication deficient recombinant adenovirus vector will be used to transduce epithelial cells of the large bronchi with the E1/E3 deleted type 5 adenovirus vector, Ad_{GV}CFTR.10, which encodes the human cystic fibrosis transmembrane conductance regulator (CFTR) gene. The objective of this study is to define the safety and pharmacodynamics of CFTR gene expression in airway epithelial cells following single administration of escalating doses to the vector. If single administration is determined to be safe, subjects will undergo repeat administration to localized areas of the bronchi. (Protocol #9409-085)

Appendix D-88B. Drs. Eric J. Sorscher and James L. Logan of the University of Alabama, Birmingham, Alabama, may conduct gene transfer experiments on 9 subjects (≥18 years of age) with cystic fibrosis (CF). The normal human cystic fibrosis transmembrane conductance regulator (CFTR) gene will be expressed by a plasmid DNA vector, pKCTR, driven by the simian virus-40 (SV40) early gene promoter. The CFTR DNA construct will be delivered by cationic liposome-based gene transfer to nasal epithelial cells. The objectives of the study are to: (1) evaluate the safety of lipid-mediated gene transfer to nasal epithelial cells (including local inflammation and mucosal tissue); and (2) evaluate efficacy as determined by correction of the chloride ion transport defect, and wild-type CFTR mRNA and protein expression. (Protocol #9312-066)

Appendix D-89. Dr. Steven M. Albelda of the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania, may conduct gene transfer experiments on 12 subjects with advanced mesothelioma. The adenovirus vector encoding the *Herpes simplex* virus thymidine kinase (HSV-TK) gene, H5.020RSVTK, will be administered through a chest tube to the pleural cavity. Tumor biopsies will be assayed for gene transfer and expression. Subjects will be monitored for immunological responses to the adenovirus vector. Ganciclovir will be administered intravenously 14 days following vector administration. The primary objective of this Phase I study is to evaluate the safety of direct adenovirus vector gene delivery to the pleural cavity of patients with malignant melanoma. (Protocol #9409-090)

Appendix D-90. Drs. Jeffrey Holt and Carlos B. Arteaga of the Vanderbilt University, Nashville, Tennessee, may conduct gene transfer experiments on 10 female patients (over 18 years of age) with metastatic breast cancer. Patient effusions from pleura or peritoneum will be drained and the fluid will be replaced with supernatant containing the retroviral vectors, XM6:antimyc or XM6:antifos, which express c-*myc* and c-*fos* antisense sequences, respectively, under the control of a mouse mammary tumor virus promoter. The objectives of this study are to: (1) assess uptake and expression of the vector sequences in breast cancer cells present in pleural and peritoneal fluids, and determine if this expression is tumor specific, (2) assess the safety of localized administration of antisense retroviruses, and (3) monitor subjects for clinical evidence of antitumor response. (Protocol #9409-084)

Appendix D-91. Dr. Jack A. Roth of MD Anderson Cancer Center, Houston, Texas, may conduct gene transfer experiments on 14 non-small cell lung cancer subjects (≥ 18 and ≤ 80 years of age) who have failed conventional therapy and who have bronchial obstruction. LNSX-based retroviral vectors containing the β-actin promoter will be used to express: (1) the antisense RNA of the K-*ras* oncogene (LN-K-*ras*B), and (2) the wildtype *p53* tumor suppressor gene (LN*p53*B). Tumor biopsies will be obtained to characterized K-*ras* and *p53* mutations. Relative to their specific mutation, subjects will undergo partial endoscopic resection of the tumor bed followed by bronchoscopic administration of the appropriate retrovirus construct. The objective of this study is to evaluate the safety and efficacy of intralesional administration of LN-K-*ras*B and LN*p53* retrovirus constructs. (Protocol #9403-031)

Appendix D-92. Drs. Robert E. Sobol and Ivor Royston of the San Diego Regional Cancer Center, San Diego, California, may conduct gene transfer experiments on 12 subjects (≥ 18 years of age) with metastatic colon carcinoma. The autologous skin fibroblasts will be transduced with the retroviral vector, LNCX/IL-2, which encodes the gene for human interleukin-2 (IL-2). In this dose-escalation study, subjects will receive subcutaneous injections of lethally irradiated autologous tumor cells. The objectives of the study are to: (1) evaluate the safety of subcutaneous administration of LNCX/IL-2 transduced fibroblasts, (2) determine *in vivo* antitumor activity, and (3) monitor cellular and humoral antitumor responses. (Protocol #9312-060)

Appendix D-93. Dr. Michael Lotze of the University of Pittsburgh, Pittsburgh, Pennsylvania, may conduct gene transfer experiments on 18 subjects (≥ 18 years of age) with advanced melanoma, 6 with T-cell lymphoma, breast cancer, or head and neck cancer. Subjects should have accessible cutaneous tumors, and have failed standard therapy. Over 4 weeks, subjects will receive a total of 4 intratumoral injections of autologous fibroblasts transduced with the retrovirus vector, TFG-hIL-12-Neo. This vector, which consists of the murine MFG backbone, expresses both the p35 and p40 subunits of interleukin-12 (IL-12) and the *neo*^{R} selection marker. The objectives of the study are to: (1) define the local and systemic toxicity associated with peritumoral injections of gene-modified fibroblasts, (2) examine the local and systemic immunomodulatory effects of these injections, and (3) evaluate clinical antitumor efficacy. (Protocol #9406-081)

Appendix D-94. Drs. Evan Hersh, Emmanuel Akporiaye, David Harris, Alison Stopeck, Evan Unger, James Warneke, of the Arizona Cancer Center, Tucson, Arizona, may conduct gene transfer experiments on 25 subjects (≥ 18 years of age) with solid malignant tumors or lymphomas. A plasmid DNA/lipid complex designated as VCL-1102 (IL-2 Plasmid DNA/DMRIE/DOPE) will be used to transduce the human gene for interleukin-2 (IL-2). Patients with advanced cancer who have failed conventional therapy will undergo a procedure in which VCL-1102 is injected directly into the tumor mass to induce tumor-specific immunity. The objectives of the study are to: (1) determine safety and toxicity associated with escalating doses of VCL-1102; (2) confirm IL-2 expression in target cells; (3) determine biological activity and pharmacokinetics; and (4) determine whether IL-2 expression stimulates tumor regression in subjects with metastatic malignancies. (Protocol #9412-095)

Appendix D-95. Drs. Richard Morgan and Robert Walker of the National Institutes of Health, Bethesda, Maryland, may conduct gene transfer experiments on 48 human immunodeficiency virus (HIV) seropositive subjects (≥ 18 years of age). This Phase I/II study involves identical twins (one HIV seropositive and the other HIV seronegative). CD4(+) T cells will be enriched following apheresis of the HIV seronegative twin, induced to polyclonal proliferation with anti-CD3 and recombinant IL-2, transduced with either the LNL6/Neo^{R} or G1Na/Neo^{R}, and transduced with up to 2 additional retroviral vectors (G1RevTdSN and/or GCRTdSN(TAR)) containing potentially therapeutic genes (antisense TAR and/or transdominant Rev). These T cell populations will be expanded 10 to 1,000 fold in culture for 1 to 2 weeks and reinfused into the HIV seropositive twin. Subjects will receive up to 4 cycles of treatment using identical or different combinations of control and anti-HIV retrovirus vectors. The relative survival of these transduced T cell populations will be monitored by vector-

specific polymerase chain reaction, while the subjects' functional immune status is monitored by standard *in vitro* and *in vivo* assays. (Protocol #9503-103)

Appendix D-96. Dr. Harry L. Malech of the National Institutes of Health, Bethesda, Maryland, may conduct gene transfer experiments on 2 subjects ≥ 18 years of age (with or without concurrent serious infection), and 3 subjects ≥ 18 years of age (with or without concurrent serious infection) or minors 13-17 years of age who have concurrent serious infection who have chronic granulomatous disease (CGD). CGD is an inherited immune deficiency disorder in which blood neutrophils and monocytes fail to produce antimicrobial oxidants ($p47^{phox}$ mutation) resulting in recurrent life-threatening infections. Subjects will undergo CD34(+) mobilization with granulocyte colony stimulating factor (G-CSF). These CD34(+) cells will be transduced with the retrovirus vector, MFG-S-$p47^{phox}$, which encodes the gene for normal $p47^{phox}$. The objectives of this study are to: (1) determine the safety of administering MFG-S-$p47^{phox}$ transduced CD34(+) cells, and (2) demonstrate increased functional oxidase activity in circulating neutrophils. (Protocol #9503-104)

Appendix D-97. Drs. Chris Evans and Paul Robbins of the University of Pittsburgh, Pittsburgh, Pennsylvania, may conduct gene transfer experiments on 6 subjects (≥ 18 and ≤ 76 years of age) with rheumatoid arthritis. Rheumatoid arthritis is a chronic, progressive disease thought to be of autoimmune origin. A gene encoding an interleukin-1 receptor antagonist protein (IRAP) will be delivered to the rheumatoid metacarpal-phalangeal joints to determine the autoimmune reactions can be interrupted. The vector construct, DFG-IRAP, is based on the MFG murine retrovirus vector backbone, and encodes the human IRAP gene. Synovial fibroblasts will be generated from the rheumatoid arthritic joint tissue obtained from patients who are scheduled to undergo surgery. The fibroblasts will be transduced with the DFG-IRAP vector, and the transduced cells injected into the synovial space. The synovial fluid and joint material will be collected 7 days later to determine the presence and location of the transduced synovial fibroblasts and the level of IRAP in the joint fluid. (Protocol 9406-074)

Appendix D-98. Dr. R. Scott McIvor of the University of Minnesota, Minneapolis, Minnesota, may conduct gene transfer experiments on 2 children with purine nucleoside phosphorylase (PNP) deficiency. PNP deficiency results in severe T-cell immunodeficiency, an autosomal recessive inherited disease which is usually fatal in the first decade of life. Autologous peripheral blood lymphocytes will be cultured in an artificial capillary cartridge in the presence of anti-CD3 monoclonal antibody and interleukin-2 and transduced with the retroviral vector, LPNSN-2, encoding human PNP. Subjects will undergo bimonthly intravenous administration of transduced T cells for a maximum of 1 year. The objectives of the study are to determine: (1) the safety of intravenous administration of transduced T cells in children with PNP deficiency, (2) the efficiency of PNP gene transfer and duration of gene expression *in vivo*, and (3) the effect of PNP gene transfer on immune function. (Protocol #9506-110)

Appendix D-99. Drs. Nikhil C. Munshi and Bart Barlogie of the University of Arkansas School for Medical Sciences, Little Rock, Arkansas, may conduct gene transfer experiments on 21 subjects (>18 and <65 years of age) with relapsed or persistent multiple myeloma who are undergoing T cell depleted allogeneic bone marrow transplantation. Donor peripheral blood lymphocytes will be cultured in vitro with interleukin-2 and anti-CD3 monoclonal antibody. T cell depleted lymphocytes will be transduced with the retroviral construct, G1Tk1SvNa.7, which encodes the Herpes simplex virus thymidine kinase (HSV-TK) gene. The transduced cells will be reinfused. In this dose escalation study, 3 subjects will undergo cell-mediated gene transfer per cohort (maximum of 5 cohorts) until Grade III or IV Graft versus Host Disease (GVHD) is observed. A maximum of 6 additional patients may be entered at that maximum tolerated dose. The objectives of this study are to determine the: (1) safety of transduced donor cell infusions, (2) effectiveness of donor cell infusions in decreasing the effects of severe GVHD, (3) effectiveness of donor cell infusions in prolonging multiple myeloma remission, and (4) effectiveness of ganciclovir in eliminating donor cells for the purpose of preventing the depletion of erythrocytes. (Protocol #9506-107)

Appendix D-100. Dr. Wayne A. Marasco of Dana-Farber Cancer Institute, Boston, Massachusetts, may conduct gene transfer experiments on 6 subjects (≥ 18 and ≤ 65 years of age) with human immunodeficiency virus type-1 (HIV-1). Autologous lymphocytes from asymptomatic subjects will be transduced *ex vivo* with a retroviral vector, LNCs105, encoding the sFv105 antibody specific for the HIV-1 envelope protein. An identical aliquot will be simultaneously transduced with a control retroviral vector lacking the sFv105 cassette. Transduced cells will be reinfused into patients and the differential survival of both populations of CD4+ lymphocytes compared. The objective of the study is to determine whether the intracellular expression of a human single chain antibody against HIV-1 envelope glycoprotein gp160 that blocks gp160 processing and the production of infectious virions can safely prolong the survival of CD4(+) lymphocytes in HIV-1-infected subjects. (Protocol #9506-111)

Appendix D-101. Dr. Henry Dorkin of the New England Medical Center, Boston, Massachusetts, and Dr. Allen Lapey of Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, propose to conduct gene transfer experiments on 16 subjects (≥ 18 years of age). An E1/partial E4-deleted, replication-deficient, type 2 adenovirus vector, AD2/CFTR-2, will be used to deliver the human cystic fibrosis transmembrane conductance regulator (CFTR) gene by aerosol administration (nebulization) to the lung of CF patients. Aerosol administration will be initiated only after initial safety data has been obtained from the lobar administration protocol (#9409-091). This is a single administration dose-escalation study in which subjects will receive between 8×10^6 and 2.5×10^{10} pfu. Subjects will be assessed for evidence of adverse, systemic, immune, inflammatory, or respiratory effects in response to AD2/CFTR-2. Subjects will be monitored for virus shedding and transgene expression. Health care workers present in the facility will be required to sign an Informed Consent document regarding the possibility of virus transmission. (Protocol #9412-074)

Appendix D-102. Drs. Charles J. Link and Donald Moorman of the Human Gene Therapy Research Institute, Des Moines, Iowa, may conduct gene transfer experiments on 24 female subjects (≥ 18 years of age) with refractory or recurrent ovarian cancer. Subjects will undergo intraperitoneal delivery (via Tenkhoff catheter) of the vector producing cells (VPC), PA317/LTKOSN.2. These VPC express the *Herpes simplex* virus thymidine kinase (HSV-TK) gene which confers sensitivity to killing by the antiviral drug, ganciclovir (GCV). The LTKOSN.2 retrovirus vector is based on the LXSN backbone. Two weeks following intraperitoneal delivery of the VPC, subjects will receive 5 mg/kg intravenous GCV twice daily for 14 days. Subjects will receive between 1×10^5 and 1×10^8 VPC/kg in this dose escalation study. Subjects will be evaluated by X-ray and peritoneoscopy of the abdomen for evidence of clinical response. The objectives of this study are to determine the safety of intraperitoneal VPC administration. (Protocol #9503-100)

Appendix D-103. Dr. David T. Curiel of the University of Alabama, Birmingham, Alabama, may conduct gene transfer experiment of 15 subjects (≥ 18 years of age) with metastatic colorectal cancer. Subjects will receive intramuscular injection of the polynucleotide vaccine, pGT63, which is a plasmid DNA vector expressing carcinoembryonic antigen (CEA) and hepatitis B surface antigen (HBsAg). The objectives of the study are to: (1) characterize the immune response to CEA and HBsAg following a single intramuscular injection and following 3 consecutive intramuscular injections, and (2) determine the safety of intramuscular injection of the plasmid DNA vector at doses ranging between 0.1 to 1.0 milligrams (single dose) and 0.9 to 3.0 milligrams (total multidose). (Protocol #9506-073)

Appendix D-104. Dr. Chester B. Whitley of the University of Minnesota, Minneapolis, Minnesota, may conduct gene transfer experiments on two adult subjects (18 years of age or older) with mild Hunter syndrome (Mucopolysaccharidosis Type II). The autologous peripheral blood lymphocytes will be transduced *ex vivo* with the retroviral vector, L2SN, encoding the human cDNA for iduronate-2-sulfatase (IDS). The transduced lymphocytes will be reinfused into the patients on a monthly basis. The study will determine the frequency of peripheral blood lymphocyte transduction and the half-life of the infused cells. Evaluation of patients will include measurement of blood levels of IDS enzyme, assessment of metabolic correction by urinary glycosaminoglycan levels, clinical response of the disease, and monitoring for potential toxicity. This Phase I study is to demonstrate the safety of the L2SN-mediated gene therapy and to provide a preliminary evaluation of clinical efficacy. (Protocol #9409-087)

Appendix D-105. Drs. James Economou, John Glaspy, and William McBride of the University of California, Los Angeles, California, may conduct gene transfer experiments on 25 subjects (≥ 18 years of age) with metastatic melanoma. The protocol is an open label, Phase I trial to evaluate the safety and immunological effects of administering lethally irradiated allogeneic and autologous melanoma cells transduced with the retroviral vector, IL-7/HyTK, which encodes the gene for human interleukin-7 (IL-7). Subjects will receive 1×10^7 irradiated unmodified autologous tumor cells in combination with escalating doses of IL-7/HyTK transduced allogeneic melanoma cells (M24 cell line). The number of M24 cells administered will be adjusted based on the level of IL-7 expression. Subjects will receive 3 biweekly subcutaneous injections of M24 cells expressing 10, 100, or 1000 nanograms of IL-7/hour *in vivo*. A final cohort of 5 subjects will receive IL-7/HyTK transduced autologous cells. Subjects will be monitored for antitumor activity by skin tests, biopsy analysis, tumor-specific antibody activity, and cytotoxic T lymphocyte precursor evaluation. Non-immunologic parameters will also be monitored. (Protocol #9503-101)

Appendix D-106. Dr. Jack A. Roth, MD Anderson Cancer Center, may conduct gene transfer experiments on 42 subjects (≥ 18 years of age) with refractory non-small cell lung cancer (NSCLC). Subjects will receive direct intratumoral injection of a replication-defective type 5 adenovirus vector, AD5CMV-p53, to deliver the normal

human *p53* tumor suppressor gene. The E1 region of AD5CMV-p53 has been replaced with a *p53* expression cassette containing the human cytomegalovirus promoter (CMV). Subjects will be divided into 2 treatment groups: (1) 21 subjects will receive Ad5CMV-p53 alone, and (2) 21 subjects will receive Ad5CMV-p53 in combination with cisplatin. Following vector administration, subjects will be isolated for 96 hours during which time, assays will be conducted to demonstrate the lack of shedding of adenovirus vector. The objectives of this study are determine: (1) the maximum tolerated dose of AD5CMV-p53, (2) qualitative and quantitative toxicity related to vector administration, and (3) biologic activity.

Prior to administration, adenovirus vector stocks will be screened for p53 mutants using the SAOS osteosarcoma cell assay that was submitted by Dr. Roth on June 23, 1995. This biologic assay compares the activity of a standard stock of Adp53 vector to the activity of newly produced stocks. The standard stock of Adp53 will be defined as mediating cell death in 100% of SAOS cells (human osteosarcoma cell line with homozygous p53 deletion) at an MOI of 50:1 (titer > 5×10^{10}) on day 5 of culture. The sensitivity of the assay for detecting inactive (presumed mutant) Adp53 vector will be determined by adding increasing amounts of Adluc (control adenovirus vector containing the luciferase gene) to the Adp53 stock to determine the percentage of inactive vector required to decrease growth inhibition of SAOS cells mediated by Adp53. The test lot of Adp53 will be tested for its ability to inhibit SAOS in a 5 day assay. Significant loss of inhibitory activity compared with the standard would indicate an unacceptable level of inactive (presumed mutant) vector. (Protocol #9406-079)

Appendix D-107A. Dr. Gary Clayman. M.D. Anderson Cancer Center, Houston, Texas, may conduct gene transfer experiments on 21 subjects (≥ 18 years of age) with refractory squamous cell carcinoma of the head and neck. Subjects will receive direct intratumoral injection of a replication-defective type 5 adenovirus vector, AD5CMV-p53, to deliver the normal human *p53* tumor suppressor gene. The E1 region of AD5CMV-p53 has been replaced with a *p53* expression cassette containing the human cytomegalovirus promoter (CMV). Subjects will be divided into 2 treatment groups: (1) those with non-resectable tumors, and (2) those with surgically accessible tumors. Subjects will receive multiple injections of vector in each dose-escalation cohort. Following vector administration, subjects will be isolated for 48 hours during which time, assays will be conducted to demonstrate the lack of shedding of adenovirus vector. The objectives of the study are to determine: (1) the maximum tolerated dose of AD5CMV-p53, (2) qualitative and quantitative toxicity related to vector administration, and (3) biologic activity.

Prior to administration, adenovirus vector stocks will be screened for p53 mutants using the SAOS osteosarcoma cell assay that was submitted by Dr. Roth on June 23, 1995. This biologic assay compares the activity of a standard stock of Adp53 vector to the activity of newly produced stocks. The standard stock of Adp53 will be defined as mediating cell death in 100% of SAOS cells (human osteosarcoma cell line with homozygous p53 deletion) at an MOI of 50:1 (titer > 5×10^{10}) on day 5 of culture. The sensitivity of the assay for detecting inactive (presumed mutant) Adp53 vector will be determined by adding increasing amounts of Adluc to the Adp53 stock to determine the percentage of inactive vector required to decrease growth inhibition of SAOS cells mediated by Adp53. The test lot of Adp53 will be tested for its ability to inhibit SAOS in a 5 day assay. Significant loss of inhibitory activity compared with the standard would indicate an unacceptable level of inactive (presumed mutant) vector. (Protocol #9412-096)

Appendix D-107B. Drs. Bernard A. Fox and Walter J. Urba of Earle A. Chiles Research Institute, Providence Medical Center, Portland, Oregon, may conduct gene transfer experiments on 18 subjects (≥ 18 years of age) with metastatic renal cell carcinoma or melanoma. Autologous tumor cells will be surgically removed, transduced *in vitro* with the cationic liposome plasmid vector, VCL-1005, which encodes human leukocyte antigen (HLA)-B7 and beta-2 microglobulin. Subjects will receive subcutaneous injection of lethally irradiated transduced cells in one limb. The contralateral limb will be injected with lethally irradiated untransduced tumor cells in combination with Bacille Calmette-Guerin (BCG). Approximately 21 days following tumor cell injection, subjects will undergo lymphadenectomy for subsequent in vitro expansion of anti-CD3 activated lymphocytes. Activated lymphocytes will be adoptively transferred on approximately day 35 in combination with a 5-day course of interleukin-2 (IL-2). On approximately day 45, subjects will receive a second cycle of IL-2. The objectives of this study are to determine: (1) the safety of administering anti-CD3 activated antitumor effector T cells in draining lymph nodes, and (2) whether HLA-B7/β-2 gene transfer augments the sensitization of anti-tumor effector T-cells in draining lymph nodes. (Protocol 9506-108)

Appendix D-108. Dr. Mitchell S. Steiner, University of Tennessee, Memphis, Tennessee, and Dr. Jeffrey T. Holt, Vanderbilt University School of Medicine, Nashville, Tennessee, may conduct gene transfer experiments on 15 male subjects (35 to 75 years of age) with metastatic prostate cancer. Malignant cells obtained from

advanced prostate cancer subjects have been demonstrated to express high levels of the protooncogene c-myc *in vivo*. The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) is expressed at high levels in prostate tissue. Following removal of malignant cells via biopsy, subjects will receive a single transrectal ultrasound-guided intraprostate quadrant injection of the retrovirus vector, XM6:MMTV-antisense c-myc, for 4 consecutive days at the site of the original biopsy. The objectives of this Phase I study are to: (1) quantitatively assess the uptake and expression of XM6:MMTV-antisense c-myc by prostate cancer cells *in vivo*, (2) determine whether c-myc gene expression is prostate tumor-specific, (3) assess safety of intraprostate injection of XM6:MMTV-antisense c-myc, and (4) biologic efficacy (antisense inhibition of tumor growth). (Protocol #9509-123)

Appendix D-109. Drs. Ronald G. Crystal, Edward Hershowitz, and Michael Lieberman, New York Hospital-Cornell Medical Center, New York, New York, may conduct gene transfer experiments on 18 subjects (18 to 70 years of age) with metastatic colon carcinoma with liver metastases. In this Phase I dose-escalation study, subjects will receive computed tomography (CT)-guided intratumoral injections of the adenovirus vector, Ad_{GV}CD.10, into the same hepatic metastasis in 4 equal volumes (100 microliters), each with a separate entry into the liver. This dosage schedule will be performed on Days 1 and 7. 5-fluorocytosine (200 milligrams/kilogram/24 hours) will be administered orally in 4 equal doses starting on day 2 and continuing through the time of laparotomy. The objectives of this study are to: (1) determine the dose-dependent toxicity of direct administration of Ad_{GV}CD.10 to hepatic metastases combined with oral administration of 5-fluorocytosine, (2) quantitatively assess transfer and expression of the cytosine deaminase gene in target cells, and (3) determine the biologic effects of direct AD_{GV}CD.10 administration to hepatic metastases. (Protocol #9509-125)

Appendix D-110. Drs. Andres Berchuck and H. Kim Lyerly of Duke University Medical Center, Durham, North Carolina, may conduct gene transfer experiments on 18 subjects (≥ 18 years of age) with refractory metastatic ovarian cancer. Autologous tumor cells obtained from ascites or surgically removed tumor will be transduced with the cationic liposome vector, PMP6A-IL2, that contains an adeno-associated virus derived plasmid DNA, a cytomegalovirus (CMV) promoter, and interleukin-2 (IL-2) complementary DNA (cDNA). In this dose-escalation study, subjects will undergo 4 cycles of intradermal injections (thigh or abdomen) of *ex vivo* transduced, lethally irradiated tumor cells in an attempt to induce an antitumor response. The objectives of the study are to evaluate: (1) the safety of intradermally injected transduced cells, and (2) antitumor response following therapy. (Protocol #9506-110)

Appendix D-111. Drs. Stephen L. Eck and Jane B. Alavi of the University of Pennsylvania Medical Center, Philadelphia, Pennsylvania, may conduct gene transfer experiments on 18 subjects (>18 years of age) with malignant glioma. The adenovirus vector encoding the *Herpes simplex* virus thymidine kinase (HSV-TK) gene, H5.020RSVTK, will be injected by a stereotactic guided technique into brain tumors. Afterwards, the patients will receive systemic ganciclovir (GCV) treatment. Patients eligible to undergo a palliative debulking procedure will receive the same treatment followed by resection on day 7, and a second dose of the vector intra-operatively. Brain tissues removed by resection will be analyzed for adenovirus infection, transgene expression, and signs of inflammation. The size and metabolic activity of tumors will be monitored by scanning with magnetic resonance imaging and positron emission tomography. The objective of the study is to evaluate the overall safety of this treatment and to gain insight into the parameters that may limit the general applicability of this approach. (Protocol #9409-089)

Appendix D-112. Drs. Robert Grossman and Savio Woo of the Baylor College of Medicine & Methodist Hospital, Houston, Texas, may conduct gene transfer experiments on 20 subjects (≥ 18 years of age) with refractive central nervous system malignancies. Subjects will receive stereotaxic injections of a replication-defective, type 5 E1/E3-deleted adenovirus vector, ADV/RSV-tk, to deliver the *Herpes simplex* virus thymidine kinase (HSV-TK) gene to tumor cells. Expression of the HSV-TK gene is driven by a Rous sarcoma virus long terminal repeat (RSV-LTR). Subjects will receive a single time-course of intravenous ganciclovir (GCV) (14 consecutive days) following vector administration. Following demonstration of safety with the initial starting dose of 1×10^8 particles in 5 subjects, additional cohorts will receive between 5×10^8 and 1.5×10^9 particles. Each cohort will be monitored for toxicity for one month before administration of the next higher dose to subsequent cohorts. Subjects will be monitored for evidence of clinical efficacy by magnetic resonance imaging and/or computer tomography scans. The primary objective of this Phase I study is to determine the safety of vector administration. (Protocol #9412-098)

Appendix D-113. Drs. Gabriel N. Hortobagyi, Gabriel Lopez-Berestein, and Mien-Chie Hung, of the University of Texas MD Anderson Cancer Center, Houston, Texas, may conduct gene transfer experiments on a maximum of 24 adult patients (12 for each cancer) with metastatic breast or ovarian carcinoma. Overexpression of the

HER-2/*neu* oncogene occurs in 30% of ovarian and breast cancers, and it is associated with enhanced metastatic potential, drug resistance, and poor survival. The E1A gene of the adenovirus type 5 functions as a tumor suppressor gene when transfected into cancer cells which overexpress the HER-2/*neu* oncogene. E1A expression induces down regulation of the level of the HER-2/*neu* oncoprotein by a transcriptional control mechanism. A plasmid, pE1A, encoding the adenovirus E1A gene with its own promoter will be administered as a DNA/lipid complex via the intraperitoneal or intrapleural route. The objectives of the study are: (1) to determine E1A gene transduction into malignant cells after the administration of E1A/lipid complex by intrapleural or intraperitoneal administration, (2) to determine whether E1A gene therapy can down-regulate HER-2/*neu* expression after intrapleural or intraperitoneal administration, (3) to determine the maximum biologically active dose (MBAD), or the maximum tolerated dose (MTD) of E1A/lipid complex, (4) to determine the toxicity and tolerance of E1A/lipid complex administered into the pleural or peritoneal space, and to assess the reversibility of such toxicity, and (5) to evaluate tumor response. (Protocol #9512-137)

Appendix D-114. Drs. Keith L. Black and Habib Fakhrai of the University of California, Los Angeles, California, may conduct gene transfer experiments on 12 subjects (≥ 18 years of age) with glioblastoma multiform. An Epstein-Barr virus (EBV) based plasmid vector, pCEP-4/TGF- β2 antisense, encoding antisense RNA will be used to inhibit TGF- β2 production. Tumor samples obtained from the patients at the time of clinically indicated surgery will be grown in culture to establish a cell line for each patient. The patients' tumor cells will be genetically altered with the pCEP-4/TGF-β2 vector to inhibit their secretion of TGF-β. Following completion of the traditional post surgical radiation therapy, the first cohort of patients will receive, at 3 week intervals, 4 injections of 5×10^6 irradiated gene modified autologous tumor cells. Subsequently, in dose escalation studies, the second cohort will receive 1×10^7 cells, and the third cohort, 2×10^7 cells. The results of this Phase I trial will be used to assess the safety of this form of gene therapy and may provide preliminary data to evaluate the potential utility of TGF- β2 antisense gene therapy in the management of gliomas. (Protocol #9512-138)

Appendix D-115. Dr. Ronald G. Crystal of New York Hospital-Cornell Medical Center, New York, New York, may conduct gene transfer experiments on a total of 21 (with an option for an additional 5) normal males and female subjects, age ≥ 18 years. Replication-deficient adenovirus (Ad) vector previously has been used in a number of human gene therapy strategies to transfer genes *in vivo* for therapeutic purposes. The purpose of this protocol is to characterize the local (skin), systemic (blood), and distant compartment (lung) immunity in normal individuals after intradermal administration of a replication deficient Ad5-based vector, named

Ad_{GV}CD.10, carrying the gene coding for the *E. coli* enzyme, cytosine deaminase (CD). Following intradermal administration of the vector to normal individuals, the skin, blood, and lung immune responses to the Ad vector and CD transgene will be evaluated over time. This vector has been safety administered intrahepatically ten times to five individuals with colon carcinoma. No adverse effects in Protocol #9509-125 have been observed. The present protocol will yield insights into normal human immune responses to both the Ad vector, as well as to a heterologous (i.e., non-human) gene product (CD). Note: This study is designed to answer basic biological questions regarding characterization of the immune responses to such vectors that have been previously documented. (Protocol #9701-171)

Appendix D-116. Dr. Daniel Rockey at Oregon State University and Dr. Walter Stamm at the University of Washington may conduct experiments to deliberately transfer a gene encoding tetracycline resistance from *Chlamydia suis* (a swine pathogen) into *C. trachomatis* (a human pathogen). This approval is specific to Drs. Rockey and Stamm and research with these resistant organisms may only occur under the conditions as specified by the NIH Director (72 FR 61661). This approval was effective as of September 24, 2007.

Appendix D-117. Dr. David Walker at the University of Texas Medical Branch may conduct experiments to deliberately introduce a gene encoding chloramphenicol resistance into *Rickettsia conorii*. This approval is specific to Dr. Walker and research with these resistant organisms may only occur under the conditions as specified by the NIH Director (73 FR 32719). This approval was effective as of April 7, 2008.

Appendix D-118. Dr. Harlan Caldwell at the Rocky Mountain Laboratories may conduct experiments to deliberately introduce a gene encoding tetracycline resistance into *Chlamydia trachomatis* serovar L2. This approval is specific to Dr. Caldwell and research with this resistant organism may only occur under the conditions as specified by the NIH Director (76 FR 27653). This approval was effective as of April 26, 2010.

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APPENDIX E. CERTIFIED HOST-VECTOR SYSTEMS (See Appendix I, Biological Containment)

While many experiments using *Escherichia coli* K-12, *Saccharomyces cerevisiae*, and *Bacillus subtilis* are currently exempt from the *NIH Guidelines* under Section III-F, *Exempt Experiments*, some derivatives of these host-vector systems were previously classified as Host-Vector 1 Systems or Host-Vector 2 Systems. A listing of those systems follows:

Appendix E-I. *Bacillus subtilis*

Appendix E-I-A. *Bacillus subtilis* Host-Vector 1 Systems

The following plasmids are accepted as the vector components of certified *B. subtilis* systems: pUB110, pC194, pS194, pSA2100, pE194, pT127, pUB112, pC221, pC223, and pAB124. *B. subtilis* strains RUB 331 and BGSC 1S53 have been certified as the host component of Host-Vector 1 systems based on these plasmids.

Appendix E-I-B. *Bacillus subtilis* Host-Vector 2 Systems

The asporogenic mutant derivative of *Bacillus subtilis*, ASB 298, with the following plasmids as the vector component: pUB110, pC194, pS194, pSA2100, pE194, pT127, pUB112, pC221, pC223, and pAB124.

Appendix E-II. Saccharomyces cerevisiae

Appendix E-II-A. *Saccharomyces cerevisiae* Host-Vector 2 Systems

The following sterile strains of *Saccharomyces cerevisiae*, all of which have the ste-VC9 mutation, SHY1, SHY2, SHY3, and SHY4. The following plasmids are certified for use: YIp1, YEp2, YEp4, YIp5, YEp6, YRp7, YEp20, YEp21, YEp24, YIp25, YIp26, YIp27, YIp28, YIp29, YIp30, YIp31, YIp32, and YIp33.

Appendix E-III. *Escherichia coli*

Appendix E-III-A. *Escherichia coli* (EK2) Plasmid Systems

The *Escherichia coli* K-12 strain chi-1776. The following plasmids are certified for use: pSC101, pMB9, pBR313, pBR322, pDH24, pBR325, pBR327, pGL101, and pHB1. The following *Escherichia coli*/S. cerevisiae hybrid plasmids are certified as EK2 vectors when used in *Escherichia coli* chi-1776 or in the sterile yeast strains, SHY1, SHY2, SHY3, and SHY4: YIpI, YEp2, YEp4, YIp5, YEp6, YRp7, YEp20, YEp21, YEP24, YIp25, YIp26, YIp27, YIp28, YIp29, YIp30, YIp31, YIp32, and YIp33.

Appendix E-III-B. *Escherichia coli* (EK2) Bacteriophage Systems

The following are certified EK2 systems based on bacteriophage lambda:

Vector	Host
λgt *WES*λB'	DP50*sup*F
λgt *WES*λB*	DP50*sup*F
λgt Z*J vir*λB'	*Escherichia coli* K-12
λgtALO·λB	DP50*sup*F
Charon 3A	DP50 or DP50*sup*F
Charon 4A	DP50 or DP50*sup*F
Charon 16A	DP50 or DP50*sup*F
Charon 21A	DP50*sup*F
Charon 23A	DP50 or DP50*sup*F
Charon 24A	DP50 or DP50*sup*F

Escherichia coli K-12 strains chi-2447 and chi-2281 are certified for use with lambda vectors that are certified for use with strain DP50 or DP50*sup*F provided that the *su*-strain not be used as a propagation host.

Appendix E-IV. *Neurospora crassa*

Appendix E-IV-A. *Neurospora crassa* **Host-Vector 1 Systems**

The following specified strains of *Neurospora crassa* which have been modified to prevent aerial dispersion:

In1 (inositol-less) strains 37102, 37401, 46316, 64001, and 89601. Csp-1 strain UCLA37 and csp-2 strains FS 590, UCLA101 (these are conidial separation mutants).

Eas strain UCLA191 (an "easily wettable" mutant).

Appendix E-V. ***Streptomyces***

Appendix E-V-A. *Streptomyces* **Host-Vector 1 Systems**

The following *Streptomyces* species: *Streptomyces coelicolor, S. lividans, S. parvulus*, and *S. griseus.* The following are accepted as vector components of certified *Streptomyces* Host-Vector 1 systems: *Streptomyces* plasmids SCP2, SLP1.2, pIJ101, actinophage phi C31, and their derivatives.

Appendix E-VI. ***Pseudomonas putida***

Appendix E-VI-A. *Pseudomonas putida* **Host-Vector 1 Systems**

Pseudomonas putida strains KT2440 with plasmid vectors pKT262, pKT263, and pKT264.

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APPENDIX F. CONTAINMENT CONDITIONS FOR CLONING OF GENES CODING FOR THE BIOSYNTHESIS OF MOLECULES TOXIC FOR VERTEBRATES

Appendix F-I. General Information

Appendix F specifies the containment to be used for the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates. The cloning of genes coding for molecules toxic for vertebrates that have an LD_{50} of < 100 nanograms per kilograms body weight (e.g., microbial toxins such as the botulinum toxins, tetanus toxin, diphtheria toxin, *Shigella dysenteriae* neurotoxin) are covered under Section III-B-1 (*Experiments Involving the Cloning of Toxin Molecules with LD_{50} of Less than 100 Nanograms Per Kilogram Body Weight*) and require Institutional Biosafety Committee and NIH/OBA approval before initiation. No specific restrictions shall apply to the cloning of genes if the protein specified by the gene has an $LD_{50} \geq 100$ micrograms per kilograms of body weight. Experiments involving genes coding for toxin molecules with an LD_{50} of < 100 micrograms per kilograms and > 100 nanograms per kilograms body weight require Institutional Biosafety Committee approval and registration with NIH/OBA prior to initiating the experiments. A list of toxin molecules classified as to LD_{50} is available from NIH/OBA. Testing procedures for determining toxicity of toxin molecules not on the list are available from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). The results of such tests shall be forwarded to NIH/OBA, which will consult with *ad hoc* experts, prior to inclusion of the molecules on the list (see Section IV-C-1-b-(2)-(c), *Minor Actions*).

Appendix F-II. Cloning of Toxin Molecule Genes in *Escherichia coli* **K-12**

Appendix F-II-A. Cloning of genes coding for molecules toxic for vertebrates that have an LD_{50} of >100 nanograms per kilograms and <1000 nanograms per kilograms body weight (e.g., abrin, *Clostridium perfringens* epsilon toxin) may proceed under Biosafety Level (BL) 2 + EK2 or BL3 + EK1 containment conditions.

Appendix F-II-B. Cloning of genes for the biosynthesis of molecules toxic for vertebrates that have an LD_{50} of >1 microgram per kilogram and <100 microgram per kilogram body weight may proceed under BL1 + EK1 containment conditions (e.g., *Staphylococcus aureus* alpha toxin, *Staphylococcus aureus* beta toxin, ricin, *Pseudomonas aeruginosa* exotoxin A, *Bordetella pertussis* toxin, the lethal factor of *Bacillus anthracis*, the *Pasteurella pestis* murine toxins, the oxygen-labile hemolysins such as streptolysin O, and certain neurotoxins present in snake venoms and other venoms).

Appendix F-II-C. Some enterotoxins are substantially more toxic when administered enterally than parenterally. The following enterotoxins shall be subject to BL1 + EK1 containment conditions: cholera toxin, the heat labile toxins of *Escherichia coli*, *Klebsiella*, and other related proteins that may be identified by neutralization with an antiserum monospecific for cholera toxin, and the heat stable toxins of *Escherichia coli* and of *Yersinia enterocolitica*.

Appendix F-III. Cloning of Toxic Molecule Genes in Organisms Other Than *Escherichia coli* K-12

Requests involving the cloning of genes coding for toxin molecules for vertebrates at an LD_{50} of <100 nanograms per kilogram body weight in host-vector systems other than *Escherichia coli* K-12 will be evaluated by NIH/OBA in consultation with *ad hoc* toxin experts (see Sections III-B-1, *Experiments Involving the Cloning of Toxin Molecules with LD_{50} of Less than 100 Nanograms Per Kilogram Body Weight*, and IV-C-1-b-(2)-(c), *Minor Actions*).

Appendix F-IV. Specific Approvals

An updated list of experiments involving the deliberate formation of recombinant or synthetic nucleic acid molecules containing genes coding for toxins lethal for vertebrates at an LD_{50} of <100 nanograms per kilogram body weight is available from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

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APPENDIX G. PHYSICAL CONTAINMENT

Appendix G specifies physical containment for standard laboratory experiments and defines Biosafety Level 1 through Biosafety Level 4. For large-scale (over 10 liters) research or production, Appendix K (*Physical Containment for Large Scale Uses of Organisms Containing Recombinant or Synthetic Nucleic Acid Molecules*) supersedes Appendix G. Appendix K defines Good Large Scale Practice through Biosafety Level 3 - Large Scale. For certain work with plants, Appendix P (*Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Plants*) supersedes Appendix G. Appendix P defines Biosafety Levels 1 through 4 - Plants. For certain work with animals, Appendix Q (*Physical and Biological Containment for Recombinant or Synthetic Nucleic Acid Molecule Research Involving Animals*) supersedes Appendix G. Appendix Q defines Biosafety Levels 1 through 4 - Animals.

Appendix G-I. Standard Practices and Training

The first principle of containment is strict adherence to good microbiological practices (see Appendices G-III-A through G-III-J, *Footnotes and References of Appendix G*). Consequently, all personnel directly or indirectly involved in experiments using recombinant or synthetic nucleic acid molecules shall receive adequate instruction (see Sections IV-B-1-h, *Responsibilities of the Institution--General Information*, and IV-B-7-d, *Responsibilities of the Principal Investigator Prior to Initiating Research*). At a minimum, these instructions include training in aseptic techniques and in the biology of the organisms used in the experiments so that the potential biohazards can be understood and appreciated.

Any research group working with agents that are known or potential biohazards shall have an emergency plan that describes the procedures to be followed if an accident contaminates personnel or the environment. The Principal Investigator shall ensure that everyone in the laboratory is familiar with both the potential hazards of the work and the emergency plan (see Sections IV-B-7-d, *Responsibilities of the Principal Investigator Prior to Initiating Research* and IV-B-7-e, *Responsibilities of the Principal Investigator During the Conduct of the Research*). If a research group is working with a known pathogen for which there is an effective vaccine, the vaccine should be made available to all workers. Serological monitoring, when clearly appropriate, will be provided (see Section IV-B-1-f, *Responsibilities of the Institution--General Information*).

The *Laboratory Safety Monograph* (see Appendix G-III-O, *Footnotes and References of Appendix G*) and *Biosafety in Microbiological and Biomedical Laboratories* (see Appendix G-III-A, *Footnotes and References of Appendix G*) describe practices, equipment, and facilities in detail.

Appendix G-II. Physical Containment Levels

The objective of physical containment is to confine organisms containing recombinant or synthetic nucleic acid molecules and to reduce the potential for exposure of the laboratory worker, persons outside of the laboratory, and the environment to organisms containing recombinant or synthetic nucleic acid molecules. Physical containment is achieved through the use of laboratory practices, containment equipment, and special laboratory design. Emphasis is placed on primary means of physical containment which are provided by laboratory practices and containment equipment. Special laboratory design provides a secondary means of protection against the accidental release of organisms outside the laboratory or to the environment. Special laboratory design is used primarily in facilities in which experiments of moderate to high potential hazard are performed.

Combinations of laboratory practices, containment equipment, and special laboratory design can be made to achieve different levels of physical containment. Four levels of physical containment, which are designated as BL1, BL2, BL3, and BL4 are described. It should be emphasized that the descriptions and assignments of physical containment detailed below are based on existing approaches to containment of pathogenic organisms (see Appendix G-III-A, *Footnotes and References of Appendix G*). The National Cancer Institute describes three levels for research on oncogenic viruses which roughly correspond to our BL2, BL3, and BL4 levels (see Appendix G-III-C, *Footnotes and References of Appendix G*).

It is recognized that several different combinations of laboratory practices, containment equipment, and special laboratory design may be appropriate for containment of specific research activities. The *NIH Guidelines*, therefore, allow alternative selections of primary containment equipment within facilities that have been designed to provide BL3 and BL4 levels of physical containment. The selection of alternative methods of primary containment is dependent, however, on the level of biological containment provided by the host-vector system used in the experiment. Consideration will be given to other combinations which achieve an equivalent level of containment (see Sections IV-C-1-b-(1), *Major Actions* and IV-C-1-b-(2), *Minor Actions*).

Appendix G-II-A. Biosafety Level 1 (BL1) (See Appendix G-III-M, *Footnotes and References of Appendix G*)

Appendix G-II-A-1. Standard Microbiological Practices (BL1)

Appendix G-II-A-1-a. Access to the laboratory is limited or restricted at the discretion of the Principal Investigator when experiments are in progress.

Appendix G-II-A-1-b. Work surfaces are decontaminated once a day and after any spill of viable material.

Appendix G-II-A-1-c. All contaminated liquid or solid wastes are decontaminated before disposal.

Appendix G-II-A-1-d. Mechanical pipetting devices are used; mouth pipetting is prohibited.

Appendix G-II-A-1-e. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.

Appendix G-II-A-1-f. Persons wash their hands: (i) after they handle materials involving organisms containing recombinant or synthetic nucleic acid molecules and animals, and (ii) before exiting the laboratory.

Appendix G-II-A-1-g. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G-II-A-1-h. In the interest of good personal hygiene, facilities (e.g., hand washing sink, shower, changing room) and protective clothing (e.g., uniforms, laboratory coats) shall be provided that are appropriate for the risk of exposure to viable organisms containing recombinant or synthetic nucleic acid molecules.

Appendix G-II-A-2. Special Practices (BL1)

Appendix G-II-A-2-a. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leak-proof container which is closed before being removed from the laboratory.

Appendix G-II-A-2-b. An insect and rodent control program is in effect.

Appendix G-II-A-3. Containment Equipment (BL1)

Appendix G-II-A-3-a. Special containment equipment is generally not required for manipulations of agents assigned to BL1.

Appendix G-II-A-4. Laboratory Facilities (BL1)

Appendix G-II-A-4-a. The laboratory is designed so that it can be easily cleaned.

Appendix G-II-A-4-b. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G-II-A-4-c. Laboratory furniture is sturdy. Spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G-II-A-4-d. Each laboratory contains a sink for hand washing.

Appendix G-II-A-4-e. If the laboratory has windows that open, they are fitted with fly screens.

Appendix G-II-B. Biosafety Level 2 (BL2) (See Appendix G-III-N, *Footnotes and References of Appendix G*)

Appendix G-II-B-1. Standard Microbiological Practices (BL2)

Appendix G-II-B-1-a. Access to the laboratory is limited or restricted by the Principal Investigator when work with organisms containing recombinant or synthetic nucleic acid molecules is in progress.

Appendix G-II-B-1-b. Work surfaces are decontaminated at least once a day and after any spill of viable material.

Appendix G-II-B-1-c. All contaminated liquid or solid wastes are decontaminated before disposal.

Appendix G-II-B-1-d. Mechanical pipetting devices are used; mouth pipetting is prohibited.

Appendix G-II-B-1-e. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.

Appendix G-II-B-1-f. Persons wash their hands: (i) after handling materials involving organisms containing recombinant or synthetic nucleic acid molecules and animals, and (ii) when exiting the laboratory.

Appendix G-II-B-1-g. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G-II-B-1-h. Experiments of lesser biohazard potential can be conducted concurrently in carefully demarcated areas of the same laboratory.

Appendix G-II-B-2. Special Practices (BL2)

Appendix G-II-B-2-a. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leak-proof container which is closed before being removed from the laboratory.

Appendix G-II-B-2-b. The Principal Investigator limits access to the laboratory. The Principal Investigator has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory.

Appendix G-II-B-2-c. The Principal Investigator establishes policies and procedures whereby only persons who have been advised of the potential hazard and meet any specific entry requirements (e.g., immunization) may enter the laboratory or animal rooms.

Appendix G-II-B-2-d. When the organisms containing recombinant or synthetic nucleic acid molecules in use in the laboratory require special provisions for entry (e.g., vaccination), a hazard warning sign incorporating the universal biosafety symbol is posted on the access door to the laboratory work area. The hazard warning sign

identifies the agent, lists the name and telephone number of the Principal Investigator or other responsible person(s), and indicates the special requirement(s) for entering the laboratory.

Appendix G-II-B-2-e. An insect and rodent control program is in effect.

Appendix G-II-B-2-f. Laboratory coats, gowns, smocks, or uniforms are worn while in the laboratory. Before exiting the laboratory for non-laboratory areas (e.g., cafeteria, library, administrative offices), this protective clothing is removed and left in the laboratory or covered with a clean coat not used in the laboratory.

Appendix G-II-B-2-g. Animals not involved in the work being performed are not permitted in the laboratory.

Appendix G-II-B-2-h. Special care is taken to avoid skin contamination with organisms containing recombinant or synthetic nucleic acid molecules; gloves should be worn when handling experimental animals and when skin contact with the agent is unavoidable.

Appendix G-II-B-2-i. All wastes from laboratories and animal rooms are appropriately decontaminated before disposal.

Appendix G-II-B-2-j. Hypodermic needles and syringes are used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) are used for the injection or aspiration of fluids containing organisms that contain recombinant or synthetic nucleic acid molecules. Extreme caution should be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Needles should not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe following use. The needle and syringe should be promptly placed in a puncture-resistant container and decontaminated, preferably autoclaved, before discard or reuse.

Appendix G-II-B-2-k. Spills and accidents which result in overt exposures to organisms containing recombinant or synthetic nucleic acid molecules are immediately reported to the Institutional Biosafety Committee and NIH/OBA. Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix G-II-B-2-l. When appropriate, considering the agent(s) handled, baseline serum samples for laboratory and other at-risk personnel are collected and stored. Additional serum specimens may be collected periodically depending on the agents handled or the function of the facility.

Appendix G-II-B-2-m. A biosafety manual is prepared or adopted. Personnel are advised of special hazards and are required to read and follow instructions on practices and procedures.

Appendix G-II-B-3. Containment Equipment (BL2)

Appendix G-II-B-3-a. Biological safety cabinets (Class I or II) (see Appendix G-III-L, *Footnotes and References of Appendix G*) or other appropriate personal protective or physical containment devices are used whenever:

Appendix G-II-B-3-a-(1). Procedures with a high potential for creating aerosols are conducted (see Appendix G-III-O, *Footnotes and References of Appendix G*). These may include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of materials whose internal pressures may be different from ambient pressures, intranasal inoculation of animals, and harvesting infected tissues from animals or eggs.

Appendix G-II-B-3-a-(2). High concentrations or large volumes of organisms containing recombinant or synthetic nucleic acid molecules are used. Such materials may be centrifuged in the open laboratory if sealed beads or centrifuge safety cups are used and if they are opened only in a biological safety cabinet.

Appendix G-II-B-4. Laboratory Facilities (BL2)

Appendix G-II-B-4-a. The laboratory is designed so that it can be easily cleaned.

Appendix G-II-B-4-b. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G-II-B-4-c. Laboratory furniture is sturdy and spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G-II-B-4-d. Each laboratory contains a sink for hand washing.

Appendix G-II-B-4-e. If the laboratory has windows that open, they are fitted with fly screens.

Appendix G-II-B-4-f. An autoclave for decontaminating laboratory wastes is available.

Appendix G-II-C. Biosafety Level 3 (BL3) (See Appendix G-III-P, *Footnotes and References of Appendix G*)

Appendix G-II-C-1. Standard Microbiological Practices (BL3)

Appendix G-II-C-1-a. Work surfaces are decontaminated at least once a day and after any spill of viable material.

Appendix G-II-C-1-b. All contaminated liquid or solid wastes are decontaminated before disposal.

Appendix G-II-C-1-c. Mechanical pipetting devices are used; mouth pipetting is prohibited.

Appendix G-II-C-1-d. Eating, drinking, smoking, storing food, and applying cosmetics are not permitted in the work area.

Appendix G-II-C-1-e. Persons wash their hands: (i) after handling materials involving organisms containing recombinant or synthetic nucleic acid molecules, and handling animals, and (ii) when exiting the laboratory.

Appendix G-II-C-1-f. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G-II-C-1-g. Persons under 16 years of age shall not enter the laboratory.

Appendix G-II-C-1-h. If experiments involving other organisms which require lower levels of containment are to be conducted in the same laboratory concurrently with experiments requiring BL3 level physical containment, they shall be conducted in accordance with all BL3 level laboratory practices.

Appendix G-II-C-2. Special Practices (BL3)

Appendix G-II-C-2-a. Laboratory doors are kept closed when experiments are in progress.

Appendix G-II-C-2-b. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leak-proof container which is closed before being removed from the laboratory.

Appendix G-II-C-2-c. The Principal Investigator controls access to the laboratory and restricts access to persons whose presence is required for program or support purposes. The Principal Investigator has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory.

Appendix G-II-C-2-d. The Principal Investigator establishes policies and procedures whereby only persons who have been advised of the potential biohazard, who meet any specific entry requirements (e.g., immunization), and who comply with all entry and exit procedures entering the laboratory or animal rooms.

Appendix G-II-C-2-e. When organisms containing recombinant or synthetic nucleic acid molecules or experimental animals are present in the laboratory or containment module, a hazard warning sign incorporating the universal biosafety symbol is posted on all laboratory and animal room access doors. The hazard warning sign identifies the agent, lists the name and telephone number of the Principal Investigator or other responsible person(s), and indicates any special requirements for entering the laboratory such as the need for immunizations, respirators, or other personal protective measures.

Appendix G-II-C-2-f. All activities involving organisms containing recombinant or synthetic nucleic acid molecules are conducted in biological safety cabinets or other physical containment devices within the containment module. No work in open vessels is conducted on the open bench.

Appendix G-II-C-2-g. The work surfaces of biological safety cabinets and other containment equipment are decontaminated when work with organisms containing recombinant or synthetic nucleic acid molecules is finished. Plastic-backed paper toweling used on non-perforated work surfaces within biological safety cabinets facilitates clean-up.

Appendix G-II-C-2-h. An insect and rodent program is in effect.

Appendix G-II-C-2-i. Laboratory clothing that protects street clothing (e.g., solid front or wrap-around gowns, scrub suits, coveralls) is worn in the laboratory. Laboratory clothing is not worn outside the laboratory, and it is decontaminated prior to laundering or disposal.

Appendix G-II-C-2-j. Special care is taken to avoid skin contamination with contaminated materials; gloves should be worn when handling infected animals and when skin contact with infectious materials is unavoidable.

Appendix G-II-C-2-k. Molded surgical masks or respirators are worn in rooms containing experimental animals.

Appendix G-II-C-2-l. Animals and plants not related to the work being conducted are not permitted in the laboratory.

Appendix G-II-C-2-m. Laboratory animals held in a BL3 area shall be housed in partial-containment caging systems, such as Horsfall units (see Appendix G-III-K, *Footnotes and References of Appendix G*), open cages placed in ventilated enclosures, solid-wall and -bottom cages covered by filter bonnets or solid-wall and -bottom cages placed on holding racks equipped with ultraviolet in radiation lamps and reflectors.

Note: Conventional caging systems may be used provided that all personnel wear appropriate personal protective devices. These protective devices shall include at a minimum wrap-around gowns, head covers, gloves, shoe covers, and respirators. All personnel shall shower on exit from areas where these devices are required.

Appendix G-II-C-2-n. All wastes from laboratories and animal rooms are appropriately decontaminated before disposal.

Appendix G-II-C-2-o. Vacuum lines are protected with high efficiency particulate air/HEPA filters and liquid disinfectant traps.

Appendix G-II-C-2-p. Hypodermic needles and syringes are used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) are used for the injection or aspiration of fluids containing organisms that contain recombinant or synthetic nucleic acid molecules. Extreme caution should be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Needles should not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe following use. The needle and syringe should be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix G-II-C-2-q. Spills and accidents which result in overt or potential exposures to organisms containing recombinant or synthetic nucleic acid molecules are immediately reported to the Biological Safety Officer, Institutional Biosafety Committee, and NIH/OBA. Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Appropriate medical evaluation, surveillance, and treatment are provided and written records are maintained.

Appendix G-II-C-2-r. Baseline serum samples for all laboratory and other at-risk personnel should be collected and stored. Additional serum specimens may be collected periodically depending on the agents handled or the function of the laboratory.

Appendix G-II-C-2-s. A biosafety manual is prepared or adopted. Personnel are advised of special hazards and are required to read and follow the instructions on practices and procedures.

Appendix G-II-C-2-t. Alternative Selection of Containment Equipment (BL3)

Experimental procedures involving a host-vector system that provides a one-step higher level of biological containment than that specified may be conducted in the BL3 laboratory using containment equipment specified for the BL2 level of physical containment. Experimental procedures involving a host-vector system that provides a one-step lower level of biological containment than that specified may be conducted in the BL3 laboratory using containment equipment specified for the BL4 level of physical containment. Alternative combination of containment safeguards are shown in Appendix G-Table 1, *Possible Alternate Combinations of Physical and Biological Containment Safeguards*.

Appendix G-II-C-3. Containment Equipment (BL3)

Appendix G-II-C-3-a. Biological safety cabinets (Class I, II, or III) (see Appendix G-III-L, *Footnotes and References of Appendix G*) or other appropriate combinations of personal protective or physical containment devices (e.g., special protective clothing, masks, gloves, respirators, centrifuge safety cups, sealed centrifuge rotors, and containment caging for animals) are used for all activities with organisms containing recombinant or synthetic nucleic acid molecules which pose a threat of aerosol exposure. These include: manipulation of cultures and of those clinical or environmental materials which may be a source of aerosols; the aerosol challenge of experimental animals; the harvesting of infected tissues or fluids from experimental animals and embryonate eggs; and the necropsy of experimental animals.

Appendix G-II-C-4. Laboratory Facilities (BL3)

Appendix G-II-C-4-a. The laboratory is separated from areas which are open to unrestricted traffic flow within the building. Passage through two sets of doors is the basic requirement for entry into the laboratory from access corridors or other contiguous areas. Physical separation of the high containment laboratory from access corridors or other laboratories or activities may be provided by a double-doored clothes change room (showers may be included), airlock, or other access facility which requires passage through two sets of doors before entering the laboratory.

Appendix G-II-C-4-b. The interior surfaces of walls, floors, and ceilings are water resistant so that they can be easily cleaned. Penetrations in these surfaces are sealed or capable of being sealed to facilitate decontaminating the area.

Appendix G-II-C-4-c. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G-II-C-4-d. Laboratory furniture is sturdy and spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G-II-C-4-e. Each laboratory contains a sink for hand washing. The sink is foot, elbow, or automatically operated and is located near the laboratory exit door.

Appendix G-II-C-4-f. Windows in the laboratory are closed and sealed.

Appendix G-II-C-4-g. Access doors to the laboratory or containment module are self-closing.

Appendix G-II-C-4-h. An autoclave for decontaminating laboratory wastes is available preferably within the laboratory.

Appendix G-II-C-4-i. A ducted exhaust air ventilation system is provided. This system creates directional airflow that draws air into the laboratory through the entry area. The exhaust air is not recirculated to any other area of the building, is discharged to the outside, and is dispersed away from the occupied areas and air intakes. Personnel shall verify that the direction of the airflow (into the laboratory) is proper. The exhaust air from the laboratory room may be discharged to the outside without being filtered or otherwise treated.

Appendix G-II-C-4-j. The high efficiency particulate air/HEPA filtered exhaust air from Class I or Class II biological safety cabinets is discharged directly to the outside or through the building exhaust system. Exhaust air from Class I or II biological safety cabinets may be recirculated within the laboratory if the cabinet is tested and certified at least every twelve months. If the HEPA-filtered exhaust air from Class I or II biological safety cabinets is to be discharged to the outside through the building exhaust air system, it is connected to this system in a manner (e.g., thimble unit connection (see Appendix G-III-L, *Footnotes and References of Appendix G*)) that avoids any interference with the air balance of the cabinets or building exhaust system.

Appendix G-II-C-5. Biosafety Level 3 Enhanced for Research Involving Risk Group 3 Influenza Viruses.

Appendix G-II-C-5-a. Containment, Practices, and Training for Research with Risk Group 3 Influenza Viruses (BL3 Enhanced).

Appendix G-II-C-5-a-(1). In addition to standard BL3 practices, the following additional personal protective equipment and practices shall be used: (1) Powered Air-purifying Respirators (PAPR) are worn. (2) Street clothes are changed to protective suit (e.g., wrap-back disposable gown, olefin protective suit). (3) Double gloves are worn. (4) Appropriate shoe coverings are worn (e.g., double disposable shoe coverings, single disposable shoe coverings if worn with footwear dedicated to BL3 enhanced laboratory use, or impervious boots or shoes of rubber or other suitable material that can be decontaminated). (5) Showers prior to exiting the laboratory should be considered depending on risk assessment of research activities.

Appendix G-II-C-5-a-(2). As proper training of laboratory workers is an essential component of biosafety, retraining and periodic reassessments (at least annually) in BL3 enhanced practices, especially the proper use of respiratory equipment, such as PAPRs, and clothing changes is required.

Appendix G-II-C-5-a-(3). Reporting of all spills and accidents, even if relatively minor, is required as described in Appendix G-II-C-2-q.

Appendix G-II-C-5-a-(4). To avoid inadvertent cross contamination of 1918 H1N1, HPAI H5N1 or human H2N2 (1957-1968): (1) Containment facilities and practices appropriate for highest Risk Group virus shall be used at all times with lower Risk Group viruses, when studied in the same laboratory room. (2) Tissue cultures with these viruses shall be conducted at separate times (temporal spacing) in the same room. (3) Separate reagents shall be used to minimize risk of cross contamination. (4) A laboratory worker shall not perform concurrent influenza virus experiments that carry the risk of unintended reassortment among 1918 H1N1, human H2N2 (1957-1968), HPAI H5N1 and other human influenza viruses. (5) Two or more laboratory workers shall not perform within the same work area simultaneous influenza virus experiments that carry the risk of unintended segment reassortment between 1918 H1N1, or HPAI H5N1, or human H2N2 (1957-1968) and other human influenza viruses. (6) Between experiments good biosafety decontamination practices (e.g., surface and biosafety cabinet surface decontamination according to standard BL3 procedures) shall be used and there shall be a thirty minute wait period after decontamination before equipment is used for experiments with any other influenza A viruses. (7) Between experiments, in addition to decontamination of the work area, clothing changes and PAPR disinfection shall be performed prior to handling a different influenza virus in the same work area. (Shower-out capability may be required by USDA/APHIS for certain experiments with HPAI H5N1.)

Appendix G-II-C-5-a-(5). Continued susceptibility of the reassortant influenza viruses containing genes and/or segments from 1918 H1N1, HPAI H5N1, and human H2N2 (1957-1968) to antiviral agents shall be established by sequence analysis or suitable biological assays. After manipulation of genes that influence sensitivity to antiviral agents, susceptibility to these agents shall be reconfirmed.

Appendix G-II-C-5-b. Containment for Animal Research.
Guidance provided in Appendix G-II-C and Appendix Q-II-C is applicable with the following emphasis on standard BL3 or BL3-N containment or additional enhancements.

Appendix G-II-C-5-b-(1). Research with small animals shall be conducted in a class II biosafety cabinet. Small animals such as rodents (e.g. mice, hamsters, rats, guinea pigs) can be housed within a negative pressure BL3 animal suite using high-density individually vented caging (IVC) systems that independently supply high efficiency particulate air/HEPA-filtered and directional air circulation. Other animals (e.g. rabbits, ferrets) that are of a size or have growth or caging requirements that preclude the use of high-density IVC systems are to be housed in negative pressure bioisolators.

Appendix G-II-C-5-b-(2). Large animals such as non-human primates shall be housed in primary barrier environments according to BL3-N containment requirements (see Appendix Q-II-c).

Appendix G-II-C-5-b-(3). Specialized training and proven competency in all assigned practices and procedures shall be required for laboratory staff, including staff involved in animal care.

Appendix G-II-C-5-b-(4). For HPAI H5N1 research, the *NIH Guidelines* defer to USDA/APHIS recommendations for biocontainment practices for loose housed animals.

Appendix G-II-C-5-c. Occupational Health
A detailed occupational health plan shall be developed in advance of working with these agents in consultation, as needed, with individuals with the appropriate clinical expertise. In addition, the appropriate public health authority shall be consulted (e.g. local public health officials) on the plan and a mock drill of this plan shall be undertaken periodically. The plan should include an incident reporting system and laboratory workers shall report all incidents.

Appendix G-II-C-5-c-(1). Laboratory workers shall be provided with medical cards which include, at a minimum, the following information: characterization of the influenza virus to which they have been potentially exposed, and 24-hour contact numbers for the Principal Investigator and institution's occupational health care provider(s).

Appendix G-II-C-5-c-(2). A detailed occupational health plan shall include: (1) Unless there is a medical contraindication to vaccination (e.g. severe egg allergy) annual seasonal influenza vaccination as prerequisite for research to reduce risk of influenza like illness requiring isolation and tests to rule out infection with experimental virus and possible co-infection with circulating influenza strains. (2) Virus specific vaccination, if available, should be offered. (3) Reporting of all respiratory symptoms and/or fever (i.e. influenza-like illnesses). (4) 24-hour access to a medical facility that is prepared to implement appropriate respiratory isolation to prevent transmission and is able to provide appropriate antiviral agents. Real-time reverse transcription-polymerase chain reaction (RT-PCR) procedures should be used to discriminate these viruses from currently circulating human influenza viruses. For exposures to viruses containing genes from 1918 H1N1 or the HA gene from human H2N2 (1957-1968), specimens shall be sent to the CDC for testing (RT-PCR and confirmatory sequencing).

Appendix G-II-C-5-c-(3). In preparing to perform research with 1918 H1N1, human H2N2 (1957-1968), or HPAI H5N1, Principal Investigators should develop a clear plan specifying who will be contacted in the event of a potential exposure (during and after work hours) to conduct a risk assessment and make decisions as to the required response, including the need for and extent of isolation of the exposed worker. After any kind of potential exposure, a rapid risk assessment shall be performed by the Principal Investigator, health and biosafety officials and subsequent actions should depend on the appraised level of risk of respiratory infection for the individual and potential for transmission to others. A laboratory worker performing research with either an influenza virus containing the HA gene from human H2N2 or an influenza virus containing genes and/or segments from 1918 H1N1, shall be informed in advance that, in the case of a *known* laboratory exposure with a high risk for infection, e.g., involving the upper or lower respiratory tract or mucous membranes, the laboratory worker will need to be isolated in a predetermined facility, rather than home isolation, until infection can be ruled out by testing (e.g., negative RT-PCR for 1918 H1N1 or human H2N2 (1957-1968)) of appropriately timed specimens. Laboratory workers shall be informed in advance that in the case of a known laboratory exposure to highly pathogenic avian influenza H5N1 strains within the Goose/Guangdong/96-like H5 lineage with high risk for infection, they should be prepared to self isolate (for example at home) until infection can be ruled out by testing (e.g., negative RT-PCR for HPAI H5N1) of appropriately timed specimens. The action taken for other types of exposures should be based on the risk assessment. In addition, based on the risk assessment: (1) treatment with appropriate antiviral agents shall be initiated, and (2) the appropriate public health authorities shall be notified.

Appendix G-II-C-5-c-(4). Influenza-like illness. If a laboratory worker, who had recent exposure (within ten days) to influenza viruses containing the human H2N2 HA gene or any gene from the 1918 H1N1 or HPAI H5N1 viruses, or to animals exposed to such viruses, demonstrates symptoms and/or signs of influenza infection (e.g., fever/chills, cough, myalgias, headache), then the lab worker shall report by phone to the supervisor/Principal Investigator and other individuals identified in the occupational health plan. The laboratory worker shall be transported to a healthcare facility that can provide adequate respiratory isolation, appropriate medical therapy, and testing to determine whether the infection is due to a recombinant or synthetic influenza virus. The appropriate public health authorities shall be informed whenever a suspected case is isolated.

Appendix G-II-C-5-c-(5). For 1918 H1N1 research, the use of antiviral agents (e.g., oseltamivir) for pre-exposure prophylaxis shall be discussed with laboratory workers in advance including a discussion of the data on the safety of long term exposure to these agents and their ability to reduce the risk of clinical disease and the limits of the data regarding protection of close contacts and the community.

Appendix G-II-C-5-c-(6). Antiviral agents for post-exposure prophylaxis shall be provided only after medical evaluation. Home supplies shall not be provided in advance for research with 1918 H1N1 or influenza viruses containing the HA gene from human H2N2.

Appendix G-II-D. Biosafety Level 4 (BL4)

Appendix G-II-D-1. Standard Microbiological Practices (BL4)

Appendix G-II-D-1-a. Work surfaces are decontaminated at least once a day and immediately after any spill of viable material.

Appendix G-II-D-1-b. Only mechanical pipetting devices are used.

Appendix G-II-D-1-c. Eating, drinking, smoking, storing food, and applying cosmetics are not permitted in the laboratory.

Appendix G-II-D-1-d. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G-II-D-2. Special Practices (BL4)

Appendix G-II-D-2-a. Biological materials to be removed from the Class III cabinets or from the maximum containment laboratory in a viable or intact state are transferred to a non-breakable, sealed primary container and then enclosed in a non-breakable, sealed secondary container which is removed from the facility through a disinfectant dunk tank, fumigation chamber, or an airlock designed for this purpose.

Appendix G-II-D-2-b. No materials, except for biological materials that are to remain in a viable or intact state, are removed from the maximum containment laboratory unless they have been autoclaved or decontaminated before exiting the facility. Equipment or material which might be damaged by high temperatures or steam is decontaminated by gaseous or vapor methods in an airlock or chamber designed for this purpose.

Appendix G-II-D-2-c. Only persons whose presence in the facility or individual laboratory rooms is required for program or support purposes are authorized to enter. The supervisor has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory. Access to the facility is limited by means of secure, locked doors; accessibility is managed by the Principal Investigator, Biological Safety Officer, or other person responsible for the physical security of the facility. Before entering, persons are advised of the potential biohazards and instructed as to appropriate safeguards for ensuring their safety. Authorized persons comply with the instructions and all other applicable entry and exit procedures. A logbook signed by all personnel indicates the date and time of each entry and exit. Practical and effective protocols for emergency situations are established.

Appendix G-II-D-2-d. Personnel enter and exit the facility only through the clothing change and shower rooms. Personnel shower each time they exit the facility. Personnel use the air locks to enter or exit the laboratory only in an emergency.

Appendix G-II-D-2-e. Street clothing is removed in the outer clothing change room and kept there. Complete laboratory clothing (may be disposable), including undergarments, pants and shirts or jump suits, shoes, and gloves, is provided and used by all personnel entering the facility. Head covers are provided for personnel who do not wash their hair during the exit shower. When exiting the laboratory and before proceeding into the shower area, personnel remove their laboratory clothing and store it in a locker or hamper in the inner change room. Protective clothing shall be decontaminated prior to laundering or disposal.

Appendix G-II-D-2-f. When materials that contain organisms containing recombinant or synthetic nucleic acid molecules or experimental animals are present in the laboratory or animal rooms, a hazard warning sign incorporating the universal biosafety symbol is posted on all access doors. The sign identifies the agent, lists the name of the Principal Investigator or other responsible person(s), and indicates any special requirements for entering the area (e.g., the need for immunizations or respirators).

Appendix G-II-D-2-g. Supplies and materials needed in the facility are brought in by way of the double-doored autoclave, fumigation chamber, or airlock which is appropriately decontaminated between each use. After securing the outer doors, personnel within the facility retrieve the materials by opening the interior doors or the autoclave, fumigation chamber, or airlock. These doors are secured after materials are brought into the facility.

Appendix G-II-D-2-h. An insect and rodent control program is in effect.

Appendix G-II-D-2-i. Materials (e.g., plants, animals, and clothing) not related to the experiment being conducted are not permitted in the facility.

Appendix G-II-D-2-j. Hypodermic needles and syringes are used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral part of unit) are used for the injection or aspiration of fluids containing organisms that contain recombinant or synthetic nucleic acid molecules. Needles should not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe following use. The needle and syringe should be placed in a puncture-resistant container and decontaminated, preferably by autoclaving before discard or reuse. Whenever possible, cannulas are used instead of sharp needles (e.g., gavage).

Appendix G-II-D-2-k. A system is set up for reporting laboratory accidents, exposures, employee absenteeism, and for the medical surveillance of potential laboratory-associated illnesses. Spills and accidents which result in overt exposures to organisms containing recombinant or synthetic nucleic acid molecules are immediately reported to the Biological Safety Officer, Institutional Biosafety Committee, and NIH/OBA. Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Written records are prepared and maintained. An essential adjunct to such a reporting-surveillance system is the availability of a facility for quarantine, isolation, and medical care of personnel with potential or known laboratory associated illnesses.

Appendix G-II-D-2-l. Laboratory animals involved in experiments requiring BL4 level physical containment shall be housed either in cages contained in Class III cabinets or in partial containment caging systems, such as Horsfall units (see Appendix G-III-K, *Footnotes and References of Appendix G*), open cages placed in ventilated enclosures, or solid-wall and -bottom cages placed on holding racks equipped with ultraviolet irradiation lamps and reflectors that are located in a specially designed area in which all personnel are required to wear one-piece positive pressure suits.

Appendix G-II-D-2-m. Alternative Selection of Containment Equipment (BL4)

Experimental procedures involving a host-vector system that provides a one-step higher level of biological containment than that specified may be conducted in the BL4 facility using containment equipment requirements specified for the BL3 level of physical containment. Alternative combinations of containment safeguards are shown in Appendix G-Table 1, *Possible Alternate Combinations of Physical and Biological Containment Safeguards*.

Appendix G-II-D-3. Containment Equipment (BL4)

Appendix G-II-D-3-a. All procedures within the facility with agents assigned to Biosafety Level 4 are conducted in the Class III biological safety cabinet or in Class I or II biological safety cabinets used in conjunction with one-piece positive pressure personnel suits ventilated by a life-support system.

Appendix G-II-D-4. Laboratory Facilities (BL4)

Appendix G-II-D-4-a. The maximum containment facility consists of either a separate building or a clearly demarcated and isolated zone within a building. Outer and inner change rooms separated by a shower are provided for personnel entering and exiting the facility. A double-doored autoclave, fumigation chamber, or ventilated airlock is provided for passage of those materials, supplies, or equipment which are not brought into the facility through the change room.

Appendix G-II-D-4-b. Walls, floors, and ceilings of the facility are constructed to form a sealed internal shell which facilitates fumigation and is animal and insect proof. The internal surfaces of this shell are resistant to liquids and chemicals, thus facilitating cleaning and decontamination of the area. All penetrations in these structures and surfaces are sealed. Any drains in the floors contain traps filled with a chemical disinfectant of demonstrated efficacy against the target agent, and they are connected directly to the liquid waste decontamination system. Sewer and other ventilation lines contain high efficiency particulate air/HEPA filters.

Appendix G-II-D-4-c. Internal facility appurtenances, such as light fixtures, air ducts, and utility pipes, are arranged to minimize the horizontal surface area on which dust can settle.

Appendix G-II-D-4-d. Bench tops have seamless surfaces which are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G-II-D-4-e. Laboratory furniture is simple and of sturdy construction; and spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G-II-D-4-f. A foot, elbow, or automatically operated hand washing sink is provided near the door of each laboratory room in the facility.

Appendix G-II-D-4-g. If there is a central vacuum system, it does not serve areas outside the facility. In-line high efficiency particulate air/HEPA filters are placed as near as practicable to each use point or service cock. Filters are installed to permit in-place decontamination and replacement. Other liquid and gas services to the facility are protected by devices that prevent back-flow.

Appendix G-II-D-4-h. If water fountains are provided, they are foot operated and are located in the facility corridors outside the laboratory. The water service to the fountain is not connected to the back-flow protected distribution system supplying water to the laboratory areas.

Appendix G-II-D-4-i. Access doors to the laboratory are self-closing and locking.

Appendix G-II-D-4-j. Any windows are breakage resistant.

Appendix G-II-D-4-k. A double-doored autoclave is provided for decontaminating materials passing out of the facility. The autoclave door which opens to the area external to the facility is sealed to the outer wall and automatically controlled so that the outside door can only be opened after the autoclave "sterilization" cycle has been completed.

Appendix G-II-D-4-l. A pass-through dunk tank, fumigation chamber, or an equivalent decontamination method is provided so that materials and equipment that cannot be decontaminated in the autoclave can be safely removed from the facility.

Appendix G-II-D-4-m. Liquid effluent from laboratory sinks, biological safety cabinets, floors, and autoclave chambers are decontaminated by heat treatment before being released from the maximum containment facility. Liquid wastes from shower rooms and toilets may be decontaminated with chemical disinfectants or by heat in the liquid waste decontamination system. The procedure used for heat decontamination of liquid wastes is evaluated mechanically and biologically by using a recording thermometer and an indicator microorganism with a defined heat susceptibility pattern. If liquid wastes from the shower room are decontaminated with chemical disinfectants, the chemical used is of demonstrated efficacy against the target or indicator microorganisms.

Appendix G-II-D-4-n. An individual supply and exhaust air ventilation system is provided. The system maintains pressure differentials and directional airflow as required to assure flows inward from areas outside of the facility toward areas of highest potential risk within the facility. Manometers are used to sense pressure differentials between adjacent areas maintained at different pressure levels. If a system malfunctions, the manometers sound an alarm. The supply and exhaust airflow is interlocked to assure inward (or zero) airflow at all times.

Appendix G-II-D-4-o. The exhaust air from the facility is filtered through high efficiency particulate air/HEPA filters and discharged to the outside so that it is dispersed away from occupied buildings and air intakes. Within the facility, the filters are located as near the laboratories as practicable in order to reduce the length of potentially contaminated air ducts. The filter chambers are designed to allow *in situ* decontamination before filters are removed and to facilitate certification testing after they are replaced. Coarse filters and HEPA filters are provided to treat air supplied to the facility in order to increase the lifetime of the exhaust HEPA filters and to protect the supply air system should air pressures become unbalanced in the laboratory.

Appendix G-II-D-4-p. The treated exhaust air from Class I and II biological safety cabinets may be discharged into the laboratory room environment or the outside through the facility air exhaust system. If exhaust air from Class I or II biological safety cabinets is discharged into the laboratory the cabinets are tested and certified at six-month intervals. *The exhaust air from Class III biological safety cabinets is discharged, without recirculation through two sets of high efficiency particulate air/HEPA filters in series, via the facility exhaust air system.* If the treated exhaust air from any of these cabinets is discharged to the outside through the facility exhaust air system, it is connected to this system in a manner (e.g., thimble unit connection (see Appendix G-III-L, *Footnotes and References of Appendix G*)) that avoids any interference with the air balance of the cabinets or the facility exhaust air system.

Appendix G-II-D-4-q. A specially designed suit area may be provided in the facility. Personnel who enter this area shall wear a one-piece positive pressure suit that is ventilated by a life-support system. The life-support system includes alarms and emergency backup breathing air tanks. Entry to this area is through an airlock fitted with airtight doors. A chemical shower is provided to decontaminate the surface of the suit before the worker exits the area. The exhaust air from the suit area is filtered by two sets of high efficiency particulate air/HEPA filters installed in series. A duplicate filtration unit, exhaust fan, and an automatically starting emergency power source are provided. The air pressure within the suit area is greater than that of any adjacent area. Emergency lighting and communication systems are provided. All penetrations into the internal shell of the suit are sealed. A double-doored autoclave is provided for decontaminating waste materials to be removed from the suit areas.

Appendix G - Table 1. Possible Alternate Combinations Of Physical And Biological Containment Safeguards

Classification of Physical & Biological Containment	Alternate Physical Containment			Alternate Biological Containment
	Laboratory Facilities	Laboratory Practices	Laboratory Equipment	
BL3/HV2	BL3 BL3	BL3 BL3	BL3 BL4	HV2 HV1
BL3/HV1	BL3 BL3	BL3 BL3	BL3 BL2	HV1 HV2
BL4/HV1	BL4 BL4	BL4 BL4	BL4 BL3	HV1 HV2

BL - Biosafety Level
HV - Host-Vector System

Appendix G-III. Footnotes and References of Appendix G

Appendix G-III-A. *Biosafety in Microbiological and Biomedical Laboratories*, 5th edition, 2007, DHHS, Public Health Service, Centers for Disease Control and Prevention, Atlanta, Georgia, and National Institutes of Health, Bethesda, Maryland.

Appendix G-III-B. *Biosafety in Microbiological and Biomedical Laboratories*, 3rd edition, May 1993, U.S. DHHS, Public Health Service, Centers for Disease Control and Prevention, Atlanta, Georgia, and NIH, Bethesda, Maryland.

Appendix G-III-C. *National Cancer Institute Safety Standards for Research Involving Oncogenic Viruses*, U.S. Department of Health, Education, and Welfare Publication No. (NIH) 75-790, October 1974.

Appendix G-III-D. *National Institutes of Health Biohazards Safety Guide*, U.S. Department of Health, Education, and Welfare, Public Health Service, NIH, U.S. Government Printing Office, Stock No. 1740-00383, 1974.

Appendix G-III-E. A. Hellman, M. N. Oxman, and R. Pollack (eds.), *Biohazards in Biological Research*, Cold Spring Harbor Laboratory 1973.

Appendix G-III-F. N. V. Steere (ed.), *Handbook of Laboratory Safety*, 2nd edition, The Chemical Rubber Co., Cleveland, Ohio, 1971.

Appendix G-III-G. Bodily, J. L, "General Administration of the Laboratory," H. L. Bodily, E. L. Updyke, and J. O. Mason (eds.), *Diagnostic Procedures for Bacterial, Mycotic, and Parasitic Infections*, American Public Health Association, New York, 1970, pp. 11-28.

Appendix G-III-H. Darlow, H. M. (1969). "Safety in the Microbiological Laboratory," in J. R. Norris and D. W. Robbins (eds.), *Methods in Microbiology*, Academic Press, Inc., New York, pp. 169-204.

Appendix G-III-I. *The Prevention of Laboratory Acquired Infection*, C. H. Collins, E. G. Hartley, and R. Pilsworth, Public Health Laboratory Service, Monograph Series No. 6, 1974.

Appendix G-III-J. Chatigny, M. A., "Protection Against Infection in the Microbiological Laboratory: Devices and Procedures," in W. W. Umbreit (ed.), *Advances in Applied Microbiology*, Academic Press, New York, New York, 1961, 3:131-192.

Appendix G-III-K. Horsfall, F. L. Jr., and J. H. Baner, *Individual Isolation of Infected Animals in a Single Room*, J. Bact., 1940, 40, 569-580.

Appendix G-III-L. Biological safety cabinets referred to in this section are classified as Class I, Class II, or Class III cabinets. A Class I is a ventilated cabinet for personnel protection having an inward flow of air away from the operator. The exhaust air from this cabinet is filtered through a high efficiency particulate air/HEPA filter. This cabinet is used in three operational modes: (i) with a full-width open front, (ii) with an installed front closure panel (having four 6-inch diameter openings) without gloves, and (iii) with an installed front closure panel equipped with arm-length rubber gloves. The face velocity of the inward flow of air through the full-width open front is 75 feet per minute or greater. A Class II cabinet is a ventilated cabinet for personnel and product protection having an open front with inward air flow for personnel protection, and HEPA filtered mass recirculated air flow for product protection. The cabinet exhaust air is filtered through a HEPA filter. The face velocity of the inward flow of air through the full-width open front is 75 feet per minute or greater. Design and performance specifications for Class II cabinets have been adopted by the National Sanitation Foundation, Ann Arbor, Michigan. A Class III cabinet is a closed-front ventilated cabinet of gas tight construction which provides the highest level of personnel protection of all biosafety safety cabinets. The interior of the cabinet is protected from contaminants exterior to the cabinet. The cabinet is fitted with arm-length rubber gloves and is operated under a negative pressure of at least 0.5 inches water gauge. All supply air is filtered through HEPA filters. Exhaust air is filtered through two HEPA filters or one HEPA filter and incinerator before being discharged to the outside environment. National Sanitation Foundation Standard 49. 1976. Class II (Laminar Flow) Biohazard Cabinetry, Ann Arbor, Michigan.

Appendix G-III-M. Biosafety Level 1 is suitable for work involving agents of unknown or minimal potential hazard to laboratory personnel and the environment. The laboratory is not separated from the general traffic patterns in the building. Work is generally conducted on open bench tops. Special containment equipment is not required or generally used. Laboratory personnel have specific training in the procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology or a related science (see Appendix G-III-A, *Footnotes and References of Appendix G*).

Appendix G-III-N. Biosafety Level 2 is similar to Level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment. It differs in that: (1) laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists; (2) access to the laboratory is limited when work is being conducted; and (3) certain procedures in which infectious aerosols are created are conducted in biological safety cabinets or other physical containment equipment (see Appendix G-III-A, *Footnotes and References of Appendix G*).

Appendix G-III-O. Office of Research Safety, National Cancer Institute, and the Special Committee of Safety and Health Experts, *Laboratory Safety Monograph: A Supplement to the NIH Guidelines for Recombinant DNA Research*, NIH, Bethesda, Maryland 1978.

Appendix G-III-P. Biosafety Level 3 is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is conducted with indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Laboratory personnel have specific training in handling pathogenic and potentially lethal agents and are supervised by competent scientists who are experienced in working with these agents. All procedures involving the manipulation of infectious material are conducted within biological safety cabinets or other physical containment devices or by personnel wearing appropriate personal protective clothing and devices. The laboratory has special engineering and design features. It is recognized, however, that many existing facilities may not have all the facility safeguards recommended for BL3 (e.g., access zone, sealed penetrations, and directional airflow, etc.). In these circumstances, acceptable safety may be achieved for routine or repetitive operations (e.g., diagnostic procedures involving the propagation of an agent for identification, typing, and susceptibility testing) in laboratories where facility features satisfy BL2 recommendations provided the recommended "Standard Microbiological Practices," "Special Practices," and "Containment Equipment" for BL3 are rigorously followed. The decision to implement this modification of BL3 recommendations should be made only by the Principal Investigator.

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APPENDIX H. SHIPMENT

Recombinant or synthetic nucleic acid molecules contained in an organism or in a viral genome shall be shipped under the applicable regulations of the U.S. Postal Service (39 Code of Federal Regulations, Part 3); the Public Health Service (42 Code of Federal Regulations, Part 72); the U.S. Department of Agriculture (9 Code of Federal Regulations, Subchapters D and E; 7 CFR, Part 340); and/or the U.S. Department of Transportation (49 Code of Federal Regulations, Parts 171-179).

Note. A host-vector system may be proposed for certification by the NIH Director in accordance with the procedures set forth in Appendix I-II, *Certification of Host-Vector Systems*. In order to ensure protection for proprietary data, any public notice regarding a host-vector system which is designated by the institution as proprietary under Section IV-D, *Voluntary Compliance*, will be issued only after consultation with the institution as to the content of the notice (see Section IV-D-3, *Certification of Host-Vector Systems - Voluntary Compliance)*.

Appendix H-I. Host organisms or viruses will be shipped as etiologic agents, regardless of whether they contain recombinant or synthetic nucleic acid molecules, if they are regulated as human pathogens by the Public Health Service (42 Code of Federal Regulations, Part 72) or as animal pathogens or plant pests under the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (Titles 9 and 7 Code of Federal Regulations, respectively).

Appendix H-II. Host organisms and viruses will be shipped as etiologic agents if they contain recombinant or synthetic nucleic acid molecules when: (i) the recombinant or synthetic nucleic acid molecule includes the complete genome of a host organism or virus regulated as a human or animal pathogen or a plant pest; or (ii)

the recombinant or synthetic nucleic acid molecule codes for a toxin or other factor directly involved in eliciting human, animal, or plant disease or inhibiting plant growth, and is carried on an expression vector or within the host chromosome and/or when the host organism contains a conjugation proficient plasmid or a generalized transducing phage; or (iii) the recombinant or synthetic nucleic acid molecule comes from a host organism or virus regulated as a human or animal pathogen or as a plant pest and has not been adequately characterized to demonstrate that it does not code for a factor involved in eliciting human, animal, or plant disease.

Appendix H-III. Footnotes and References of Appendix H

For further information on shipping etiologic agents contact: (i) The Centers for Disease Control and Prevention, ATTN: Biohazards Control Office, 1600 Clifton Road, Atlanta, Georgia 30333, (404) 639-3883, FTS 236-3883; (ii) The U.S. Department of Transportation, ATTN: Office of Hazardous Materials Transportation, 400 7th Street, S.W., Washington, DC 20590, (202) 366-4545; or (iii) U.S. Department of Agriculture, ATTN: Animal and Plant Health Inspection Service (APHIS), Veterinary Services, National Center for Import-Export, Products Program, 4700 River Road, Unit 40, Riverdale, Maryland 20737. Phone: (301) 734-8499; Fax: (301) 734-8226.

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APPENDIX I. BIOLOGICAL CONTAINMENT (See Appendix E, *Certified Host-Vector Systems*)

Appendix I-I. Levels of Biological Containment

In consideration of biological containment, the vector (plasmid, organelle, or virus) for the recombinant or synthetic nucleic acid molecule and the host (bacterial, plant, or animal cell) in which the vector is propagated in the laboratory will be considered together. Any combination of vector and host which is to provide biological containment shall be chosen or constructed so that the following types of "escape" are minimized: (i) survival of the vector in its host outside the laboratory, and (ii) transmission of the vector from the propagation host to other non-laboratory hosts. The following levels of biological containment (host-vector systems) for prokaryotes are established. Appendices I-I-A through I-II-B describe levels of biological containment (host-vector systems) for prokaryotes. Specific criteria will depend on the organisms to be used.

Appendix I-I-A. Host-Vector 1 Systems

Host-Vector 1 systems provide a moderate level of containment. Specific Host-Vector 1 systems are:

Appendix I-I-A-1. *Escherichia coli* K-12 Host-Vector 1 Systems (EK1)

The host is always *Escherichia coli* K-12 or a derivative thereof, and the vectors include non-conjugative plasmids (e.g., pSC101, Co1E1, or derivatives thereof (see Appendices I-III-A through G, *Footnotes and References of Appendix I*) and variants of bacteriophage, such as lambda (see Appendices I-III-H through O, *Footnotes and References of Appendix I*). The *Escherichia coli* K-12 hosts shall not contain conjugation-proficient plasmids, whether autonomous or integrated, or generalized transducing phages.

Appendix I-I-A-2. Other Host-Vector 1 Systems

At a minimum, hosts and vectors shall be comparable in containment to *Escherichia coli* K-12 with a non-conjugative plasmid or bacteriophage vector. Appendix I-II, *Certification of Host-Vector Systems*, describes the data to be considered and mechanism for approval of Host-Vector 1 systems.

Appendix I-I-B. Host-Vector 2 Systems (EK2)

Host-Vector 2 Systems provide a high level of biological containment as demonstrated by data from suitable tests performed in the laboratory. Escape of the recombinant or synthetic nucleic acid molecule either via survival of the organisms or via transmission of the recombinant or synthetic nucleic acid molecule to other organisms should be $< 1/10^8$ under specified conditions. Specific Host-Vector 2 systems are:

Appendix I-I-B-1. For *Escherichia coli* K-12 Host-Vector 2 systems (EK2) in which the vector is a plasmid, no more than $1/10^8$ host cells shall perpetuate a cloned DNA fragment under the specified non-permissive laboratory conditions designed to represent the natural environment, either by survival of the original host or as a consequence of transmission of the cloned DNA fragment.

Appendix I-I-B-2. For *Escherichia coli* K-12 Host-Vector 2 systems (EK2) in which the vector is a phage, no more than $1/10^8$ phage particles shall perpetuate a cloned DNA fragment under the specified non-permissive laboratory conditions designed to represent the natural environment, either as a prophage (in the inserted or plasmid form) in the laboratory host used for phage propagation, or survival in natural environments and transferring a cloned DNA fragment to other hosts (or their resident prophages).

Appendix I-II. Certification of Host-Vector Systems

Appendix I-II-A. Responsibility

Host-Vector 1 systems (other than *Escherichia coli* K-12) and Host-Vector 2 systems may not be designated as such until they have been certified by the NIH Director. Requests for certification of host-vector systems may be submitted to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Proposed host-vector systems will be reviewed by the RAC (see Section IV-C-1-b-(1)-(f), *Major Actions*). Initial review will based on the construction, properties, and testing of the proposed host-vector system by a subcommittee composed of one or more RAC members and/or *ad hoc* experts. The RAC will evaluate the subcommittee's report and any other available information at the next scheduled RAC meeting. The NIH Director is responsible for certification of host-vector systems, following advice of the RAC. Minor modifications to existing host-vector systems (i.e., those that are of minimal or no consequence to the properties relevant to containment) may be certified by the NIH Director without prior RAC review (see Section IV-C-1-b-(2)-(f), *Minor Actions*). Once a host-vector system has been certified by the NIH Director, a notice of certification will be sent by NIH/OBA to the applicant and to the Institutional Biosafety Committee Chairs. A list of all currently certified host-vector systems is available from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). The NIH Director may rescind the certification of a host-vector system (see Section IV-C-1-b-(2)-(g), *Minor Actions*). If certification is rescinded, NIH will instruct investigators to transfer cloned DNA into a different system or use the clones at a higher level of physical containment level, unless NIH determines that the already constructed clones incorporate adequate biological containment. Certification of an host-vector system does not extend to modifications of either the host or vector component of that system. Such modified systems shall be independently certified by the NIH Director. If modifications are minor, it may only be necessary for the investigator to submit data showing that the modifications have either improved or not impaired the major phenotypic traits on which the containment of the system depends. Substantial modifications to a certified host-vector system requires submission of complete testing data.

Appendix I-II-B. Data to be Submitted for Certification

Appendix I-II-B-1. Host-Vector 1 Systems Other than *Escherichia coli* K-12

The following types of data shall be submitted, modified as appropriate for the particular system under consideration: (i) a description of the organism and vector; the strain's natural habitat and growth requirements; its physiological properties, particularly those related to its reproduction, survival, and the mechanisms by which it exchanges genetic information; the range of organisms with which this organism normally exchanges genetic information and the type of information is exchanged; and any relevant information about its pathogenicity or toxicity; (ii) a description of the history of the particular strains and vectors to be used, including data on any mutations which render this organism less able to survive or transmit genetic information; and (iii) a general description of the range of experiments contemplated with emphasis on the need for developing such an Host-Vector 1 system.

Appendix I-II-B-2. Host-Vector 2 Systems

Investigators planning to request Host-Vector 2 systems certification may obtain instructions from NIH/OBA concerning data to be submitted (see Appendices I-III-N and O, *Footnotes and References of Appendix I*). In general, the following types of data are required: (i) description of construction steps with indication of source,

properties, and manner of introduction of genetic traits; (ii) quantitative data on the stability of genetic traits that contribute to the containment of the system; (iii) data on the survival of the host-vector system under non-permissive laboratory conditions designed to represent the relevant natural environment; (iv) data on transmissibility of the vector and/or a cloned DNA fragment under both permissive and non-permissive conditions; (v) data on all other properties of the system which affect containment and utility, including information on yields of phage or plasmid molecules, ease of DNA isolation, and ease of transfection or transformation; and (vi) in some cases, the investigator may be asked to submit data on survival and vector transmissibility from experiments in which the host-vector is fed to laboratory animals or one or more human subjects. Such *in vivo* data may be required to confirm the validity of predicting *in vivo* survival on the basis of *in vitro* experiments. Data shall be submitted 12 weeks prior to the RAC meeting at which such data will be considered by the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Investigators are encouraged to publish their data on the construction, properties, and testing of proposed Host Vector 2 systems prior to consideration of the system by the RAC and its subcommittee. Specific instructions concerning the submission of data for proposed *Escherichia coli* K-12 Host-Vector 2 system (EK2) involving either plasmids or bacteriophage in *Escherichia coli* K-12, are available from the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax).

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Appendix I-III-N. Skalka, A., *Current Status of Coliphage AEK2 Vectors*, Gene, 1978, 3, pp. 29-35.

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APPENDIX J. BIOTECHNOLOGY RESEARCH SUBCOMMITTEE

The National Science and Technology Council's Committee on Fundamental Science determined that a subcommittee should be continued to identify and coordinate Federal research efforts, identify research needs, stimulating international cooperation, and assess national and international policy issues concerning biotechnology sciences. The primary emphasis will be on scientific issues to increase the overall effectiveness and productivity of the Federal investment in biotechnology sciences, especially regarding issues which cut across agency boundaries. This subcommittee is called the Biotechnology Research Subcommittee.

Membership of the Biotechnology Research Subcommittee will include Federal agencies that support biotechnology research. Agencies represented are: U.S. Department of Agriculture, Department of Commerce, Department of Defense, Department of Energy, Department of Health and Human Services, Department of Interior, Department of Justice, Department of State, Department of Veterans Affairs, Agency for International Development, Environmental Protection Agency, National Aeronautics and Space Administration, and National Science Foundation. The Biotechnology Research Subcommittee will function in an advisory capacity to the Committee on Fundamental Science, the Director of the Office of Science and Technology Policy, and the Executive Office of the President. The Biotechnology Research Subcommittee will review the scientific aspects of proposed regulations and guidelines as they are developed.

The primary responsibilities of the Biotechnology Research Subcommittee are to: (i) describe and review current Federal efforts in biotechnology research; (ii) identify and define the priority areas for future Federal biotechnology research, including areas needing greater emphasis, describing the role of each agency in those areas, and delineate where interagency cooperation would enhance progress in the biotechnology sciences, with an emphasis on integrated research efforts, where appropriate; (iii) assess major international efforts in the biotechnology sciences and develop mechanisms for international collaboration. For example, activities of the U.S.-European Community Task Force on Biotechnology have been coordinated through the Biotechnology Research Subcommittee; (iv) identify and review national and international policy issues (such as public education) associated with biotechnology; and (v) provide reviews, analyses, and recommendations to the Chairs of the Committee on Fundamental Science on scientific issues related to regulations and the applications of biotechnology research and biotechnology policies and issues.

In 1990, the Biotechnology Research Subcommittee replaced the Biotechnology Sciences Coordinating Committee. Both the Biotechnology Research Subcommittee and the Biotechnology Sciences Coordinating Committee previously functioned under the Federal Coordinating Council on Science, Engineering, and Technology (FCCSET). While regulatory issues became the primary focus of the Biotechnology Sciences Coordinating Committee, the Biotechnology Research Subcommittee focuses on scientific issues, although it will still provide scientific support for regulatory responsibilities.

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APPENDIX K. PHYSICAL CONTAINMENT FOR LARGE SCALE USES OF ORGANISMS CONTAINING RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES

Appendix K specifies physical containment guidelines for large-scale (greater than 10 liters of culture) research or production involving viable organisms containing recombinant or synthetic nucleic acid molecules. It shall apply to large-scale research or production activities as specified in Section III-D-6, *Experiments Involving More than 10 Liters of Culture*. It is important to note that this appendix addresses only the biological hazard associated with organisms containing recombinant or synthetic nucleic acid molecules. Other hazards accompanying the large-scale cultivation of such organisms (e.g., toxic properties of products; physical, mechanical, and chemical aspects of downstream processing) are not addressed and shall be considered separately, albeit in conjunction with this appendix.

All provisions shall apply to large-scale research or production activities with the following modifications: (i) Appendix K shall supersede Appendix G, *Physical Containment*, when quantities in excess of 10 liters of culture are involved in research or production. Appendix K-II applies to Good Large Scale Practice; (ii) the institution shall appoint a Biological Safety Officer if it engages in large-scale research or production activities involving viable organisms containing recombinant or synthetic nucleic acid molecules. The duties of the Biological Safety Officer shall include those specified in Section IV-B-3, *Biological Safety Officer*; (iii) the institution shall establish and maintain a health surveillance program for personnel engaged in large-scale research or production activities involving viable organisms containing recombinant or synthetic nucleic acid molecules which require Biosafety Level (BL) 3 containment at the laboratory scale. The program shall include: preassignment and periodic physical and medical examinations; collection, maintenance, and analysis of serum specimens for monitoring serologic changes that may result from the employee's work experience; and provisions for the investigation of any serious, unusual, or extended illnesses of employees to determine possible occupational origin.

Appendix K-I. Selection of Physical Containment Levels

The selection of the physical containment level required for recombinant or synthetic nucleic acid molecule research or production involving more than 10 liters of culture is based on the containment guidelines established in Section III, *Experiments Covered by the NIH Guidelines*. For purposes of large-scale research or production, four physical containment levels are established. The four levels set containment conditions at those appropriate for the degree of hazard to health or the environment posed by the organism, judged by experience with similar organisms unmodified by recombinant or synthetic nucleic acid molecule techniques and consistent with Good Large Scale Practice. The four biosafety levels of large-scale physical containment are referred to as Good Large Scale Practice, BL1-Large Scale, BL2-Large Scale, and BL3-Large Scale. Good Large Scale Practice is recommended for large-scale research or production involving viable, non-pathogenic, and non-toxigenic recombinant or synthetic strains derived from host organisms that have an extended history of safe large-scale use. Good Large Scale Practice is recommended for organisms such as those included in Appendix C, *Exemptions under Section III-F-8*, which have built-in environmental limitations that permit optimum growth in the large-scale setting but limited survival without adverse consequences in the environment. BL1-Large Scale is recommended for large-scale research or production of viable organisms containing recombinant or synthetic nucleic acid molecules that require BL1 containment at the laboratory scale and that do not qualify for Good Large Scale Practice. BL2-Large Scale is recommended for large-scale research or production of viable organisms containing recombinant or synthetic nucleic acid molecules that require BL2 containment at the laboratory scale. BL3-Large Scale is recommended for large-scale research or production of viable organisms containing recombinant or synthetic nucleic acid molecules that require BL3 containment at the laboratory scale. No provisions are made for large-scale research or production of viable organisms containing recombinant or synthetic nucleic acid molecules that require BL4 containment at the laboratory scale. If necessary, these requirements will be established by NIH on an individual basis.

Appendix K-II. Good Large Scale Practice (GLSP)

Appendix K-II-A. Institutional codes of practice shall be formulated and implemented to assure adequate control of health and safety matters.

Appendix K-II-B. Written instructions and training of personnel shall be provided to assure that cultures of viable organisms containing recombinant or synthetic nucleic acid molecules are handled prudently and that the work place is kept clean and orderly.

Appendix K-II-C. In the interest of good personal hygiene, facilities (e.g., hand washing sink, shower, changing room) and protective clothing (e.g., uniforms, laboratory coats) shall be provided that are appropriate for the risk of exposure to viable organisms containing recombinant or synthetic nucleic acid molecules. Eating, drinking, smoking, applying cosmetics, and mouth pipetting shall be prohibited in the work area.

Appendix K-II-D. Cultures of viable organisms containing recombinant or synthetic nucleic acid molecules shall be handled in facilities intended to safeguard health during work with microorganisms that do not require containment.

Appendix K-II-E. Discharges containing viable recombinant or synthetic organisms shall be handled in accordance with applicable governmental environmental regulations.

Appendix K-II-F. Addition of materials to a system, sample collection, transfer of culture fluids within/between systems, and processing of culture fluids shall be conducted in a manner that maintains employee's exposure to viable organisms containing recombinant or synthetic nucleic acid molecules at a level that does not adversely affect the health and safety of employees.

Appendix K-II-G. The facility's emergency response plan shall include provisions for handling spills.

Appendix K-III. Biosafety Level 1 (BL1) - Large Scale

Appendix K-III-A. Spills and accidents which result in overt exposures to organisms containing recombinant or synthetic nucleic acid molecules are immediately reported to the Laboratory Director. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix K-III-B. Cultures of viable organisms containing recombinant or synthetic nucleic acid molecules shall be handled in a closed system (e.g., closed vessel used for the propagation and growth of cultures) or other primary containment equipment (e.g., biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to reduce the potential for escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system or other primary containment equipment provided all physical containment requirements specified in Appendix G-II-A, *Physical Containment Levels--Biosafety Level 1*, are met.

Appendix K-III-C. Culture fluids (except as allowed in Appendix K-III-D) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant or synthetic nucleic acid molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules. Culture fluids that contain viable organisms or viral vectors intended as final product may be removed from the primary containment equipment by way of closed systems for sample analysis, further processing or final fill.

Appendix K-III-D. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of culture fluids from one closed system to another shall be conducted in a manner which minimizes the release of aerosols or contamination of exposed surfaces.

Appendix K-III-E. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or by other equivalent procedures (e.g., incineration) to minimize the release of viable organisms containing recombinant or synthetic nucleic acid molecules to the environment.

Appendix K-III-F. A closed system or other primary containment equipment that has contained viable organisms containing recombinant or synthetic nucleic acid molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure except when the culture fluids contain viable organisms or vectors intended as final product as described in Appendix K-III-C above. A validated sterilization procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules.

Appendix K-III-G. Emergency plans required by Sections IV-B-2-b-(6), *Institutional Biosafety Committee*, and IV-B-3-c-(3), *Biological Safety Officer*, shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K-IV. Biosafety Level 2 (BL2) - Large Scale

Appendix K-IV-A. Spills and accidents which result in overt exposures to organisms containing recombinant or synthetic nucleic acid molecules are immediately reported to the Biological Safety Officer, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix K-IV-B. Cultures of viable organisms containing recombinant or synthetic nucleic acid molecules shall be handled in a closed system (e.g., closed vessel used for the propagation and growth of cultures) or other primary containment equipment (e.g., Class III biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to prevent the escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system or other primary containment equipment provided all physical containment requirements specified in Appendix G-II-B, *Physical Containment Levels--Biosafety Level 2*, are met.

Appendix K-IV-C. Culture fluids (except as allowed in Appendix K-IV-D) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant or synthetic nucleic acid molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules. Culture fluids that contain viable organisms or viral vectors intended as final product may be removed from the primary containment equipment by way of closed systems for sample analysis, further processing or final fill.

Appendix K-IV-D. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of cultures fluids from one closed system to another shall be conducted in a manner which prevents the release of aerosols or contamination of exposed surfaces.

Appendix K-IV-E. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or by other equivalent procedures (e.g., incineration) to prevent the release of viable organisms containing recombinant or synthetic nucleic acid molecules to the environment.

Appendix K-IV-F. A closed system or other primary containment equipment that has contained viable organisms containing recombinant or synthetic nucleic acid molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure except when the culture fluids contain viable organisms or vectors intended as final product as described in Appendix K-IV-C above. A validated sterilization procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules.

Appendix K-IV-G. Rotating seals and other mechanical devices directly associated with a closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules shall be designed to prevent leakage or shall be fully enclosed in ventilated housings that are exhausted through filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or through other equivalent treatment devices.

Appendix K-IV-H. A closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules and other primary containment equipment used to contain operations involving viable organisms containing sensing devices that monitor the integrity of containment during operations.

Appendix K-IV-I. A closed system used for the propagation and growth of viable organisms containing the recombinant or synthetic nucleic acid molecules shall be tested for integrity of the containment features using the organism that will serve as the host for propagating recombinant or synthetic nucleic acid molecules.

Testing shall be accomplished prior to the introduction of viable organisms containing recombinant or synthetic nucleic acid molecules and following modification or replacement of essential containment features. Procedures and methods used in the testing shall be appropriate for the equipment design and for recovery and demonstration of the test organism. Records of tests and results shall be maintained on file.

Appendix K-IV-J. A closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules shall be permanently identified. This identification shall be used in all records reflecting testing, operation, and maintenance and in all documentation relating to use of this equipment for research or production activities involving viable organisms containing recombinant or synthetic nucleic acid molecules.

Appendix K-IV-K. The universal biosafety sign shall be posted on each closed system and primary containment equipment when used to contain viable organisms containing recombinant or synthetic nucleic acid molecules.

Appendix K-IV-L. Emergency plans required by Sections IV-B-2-b-(6), *Institutional Biosafety Committee*, and IV-B-3-c-(3), *Biological Safety Officer*, shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K-V. Biosafety Level 3 (BL3) - Large Scale

Appendix K-V-A. Spills and accidents which result in overt exposures to organisms containing recombinant or synthetic nucleic acid molecules are immediately reported to the Biological Safety Officer, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix K-V-B. Cultures of viable organisms containing recombinant or synthetic nucleic acid molecules shall be handled in a closed system (e.g., closed vessels used for the propagation and growth of cultures) or other primary containment equipment (e.g., Class III biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to prevent the escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system provided all physical containment requirements specified in Appendix G-II-C, *Physical Containment Levels--Biosafety Level 3*, are met.

Appendix K-V-C. Culture fluids (except as allowed in Appendix K-V-D) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant or synthetic nucleic acid molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules. Culture fluids that contain viable organisms or viral vectors intended as final product may be removed from the primary containment equipment by way of closed systems for sample analysis, further processing or final fill.

Appendix K-V-D. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of culture fluids from one closed system to another shall be conducted in a manner which prevents the release of aerosols or contamination of exposed surfaces.

Appendix K-V-E. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or by other equivalent procedures (e.g., incineration) to prevent the release of viable organisms containing recombinant or synthetic nucleic acid molecules to the environment.

Appendix K-V-F. A closed system or other primary containment equipment that has contained viable organisms containing recombinant or synthetic nucleic acid molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure except when the culture fluids contain viable organisms or vectors intended as final product as described in Appendix K-V-C above. A validated sterilization procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules.

Appendix K-V-G. A closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules shall be operated so that the space above the culture level will be maintained at a pressure as low as possible, consistent with equipment design, in order to maintain the integrity of containment features.

Appendix K-V-H. Rotating seals and other mechanical devices directly associated with a closed system used to contain viable organisms containing recombinant or synthetic nucleic acid molecules shall be designed to prevent leakage or shall be fully enclosed in ventilated housings that are exhausted through filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or through other equivalent treatment devices.

Appendix K-V-I. A closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules and other primary containment equipment used to contain operations involving viable organisms containing recombinant or synthetic nucleic acid molecules shall include monitoring or sensing devices that monitor the integrity of containment during operations.

Appendix K-V-J. A closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules shall be tested for integrity of the containment features using the organisms that will serve as the host for propagating the recombinant or synthetic nucleic acid molecules. Testing shall be accomplished prior to the introduction of viable organisms containing recombinant or synthetic nucleic acid molecules and following modification or replacement of essential containment features. Procedures and methods used in the testing shall be appropriate for the equipment design and for recovery and demonstration of the test organism. Records of tests and results shall be maintained on file.

Appendix K-V-K. A closed system used for the propagation and growth of viable organisms containing recombinant or synthetic nucleic acid molecules shall be permanently identified. This identification shall be used in all records reflecting testing, operation, maintenance, and use of this equipment for research production activities involving viable organisms containing recombinant or synthetic nucleic acid molecules.

Appendix K-V-L. The universal biosafety sign shall be posted on each closed system and primary containment equipment when used to contain viable organisms containing recombinant or synthetic nucleic acid molecules.

Appendix K-V-M. Emergency plans required by Sections IV-B-2-b-(6), *Institutional Biosafety Committee*, and IV-B-3-c-(3), *Biological Safety Officer*, shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K-V-N. Closed systems and other primary containment equipment used in handling cultures of viable organisms containing recombinant or synthetic nucleic acid molecules shall be located within a controlled area which meets the following requirements:

Appendix K-V-N-1. The controlled area shall have a separate entry area. The entry area shall be a double-doored space such as an air lock, anteroom, or change room that separates the controlled area from the balance of the facility.

Appendix K-V-N-2. The surfaces of walls, ceilings, and floors in the controlled area shall be such as to permit ready cleaning and decontamination.

Appendix K-V-N-3. Penetrations into the controlled area shall be sealed to permit liquid or vapor phase space decontamination.

Appendix K-V-N-4. All utilities and service or process piping and wiring entering the controlled area shall be protected against contamination.

Appendix K-V-N-5. Hand washing facilities equipped with foot, elbow, or automatically operated valves shall be located at each major work area and near each primary exit.

Appendix K-V-N-6. A shower facility shall be provided. This facility shall be located in close proximity to the controlled area.

Appendix K-V-N-7. The controlled area shall be designed to preclude release of culture fluids outside the controlled area in the event of an accidental spill or release from the closed systems or other primary containment equipment.

Appendix K-V-N-8. The controlled area shall have a ventilation system that is capable of controlling air movement. The movement of air shall be from areas of lower contamination potential to areas of higher contamination potential. If the ventilation system provides positive pressure supply air, the system shall operate in a manner that prevents the reversal of the direction of air movement or shall be equipped with an alarm that would be actuated in the event that reversal in the direction of air movement were to occur. The exhaust air from the controlled area shall not be recirculated to other areas of the facility. The exhaust air from the controlled area may not be discharged to the outdoors without being high efficiency particulate air/HEPA filtered, subjected to thermal oxidation, or otherwise treated to prevent the release of viable organisms.

Appendix K-V-O. The following personnel and operational practices shall be required:

Appendix K-V-O-1. Personnel entry into the controlled area shall be through the entry area specified in Appendix K-V-N-1.

Appendix K-V-O-2. Persons entering the controlled area shall exchange or cover their personal clothing with work garments such as jump suits, laboratory coats, pants and shirts, head cover, and shoes or shoe covers. On exit from the controlled area the work clothing may be stored in a locker separate from that used for personal clothing or discarded for laundering. Clothing shall be decontaminated before laundering.

Appendix K-V-O-3. Entry into the controlled area during periods when work is in progress shall be restricted to those persons required to meet program or support needs. Prior to entry, all persons shall be informed of the operating practices, emergency procedures, and the nature of the work conducted.

Appendix K-V-O-4. Persons under 18 years of age shall not be permitted to enter the controlled area.

Appendix K-V-O-5. The universal biosafety sign shall be posted on entry doors to the controlled area and all internal doors when any work involving the organism is in progress. This includes periods when decontamination procedures are in progress. The sign posted on the entry doors to the controlled area shall include a statement of agents in use and personnel authorized to enter the controlled area.

Appendix K-V-O-6. The controlled area shall be kept neat and clean.

Appendix K-V-O-7. Eating, drinking, smoking, and storage of food are prohibited in the controlled area.

Appendix K-V-O-8. Animals and plants shall be excluded from the controlled area.

Appendix K-V-O-9. An effective insect and rodent control program shall be maintained.

Appendix K-V-O-10. Access doors to the controlled area shall be kept closed, except as necessary for access, while work is in progress. Serve doors leading directly outdoors shall be sealed and locked while work is in progress.

Appendix K-V-O-11. Persons shall wash their hands when exiting the controlled area.

Appendix K-V-O-12. Persons working in the controlled area shall be trained in emergency procedures.

Appendix K-V-O-13. Equipment and materials required for the management of accidents involving viable organisms containing recombinant or synthetic nucleic acid molecules shall be available in the controlled area.

Appendix K-V-O-14. The controlled area shall be decontaminated in accordance with established procedures following spills or other accidental release of viable organisms containing recombinant or synthetic nucleic acid molecules.

Appendix K - Table 1. Comparison of Good Large Scale Practice (GLSP) and Biosafety Level (BL) - Large Scale (LS) Practice (See Appendix K-VI-A, *Footnotes Of Appendix K*)

	CRITERION [See Appendix K-VI-B, *Footnotes of Appendix K*]	GLSP	BL1-LS	BL2-LS	BL3-LS
1.	Formulate and implement institutional codes of practice for safety of personnel and adequate control of hygiene and safety measures.	K-II-A	G-I		
2.	Provide adequate written instructions and training of personnel to keep work place clean and tidy and to keep exposure to biological, chemical or physical agents at a level that does not adversely affect health and safety of employees.	K-II-B	G-I		
3.	Provide changing and hand washing facilities as well as protective clothing, appropriate to the risk, to be worn during work.	K-II-C	G-II-A-1-h	G-II-B-2-f	G-II-C-2-i
4.	Prohibit eating, drinking, smoking, mouth pipetting, and applying cosmetics in the work place.	K-II-C	G-II-A-1-d G-II-A-1-e	G-II-B-1-d G-II-B-1-e	G-II-C-1-c G-II-C-1-d
5.	Internal accident reporting.	K-II-G	K-III-A	K-IV-A	K-V-A
6.	Medical surveillance.	NR	NR		
7.	Viable organisms should be handled in a system that physically separates the process from the external environment (closed system or other primary containment).	NR	K-III-B	K-IV-B	K-V-B
8.	Culture fluids not removed from a system until organisms are inactivated.	NR	K-III-C	K-IV-C	K-V-C
9.	Inactivation of waste solutions and materials with respect to their biohazard potential.	K-II-E	K-III-C	K-IV-C	K-V-C
10.	Control of aerosols by engineering or procedural controls to prevent or minimize release of organisms during sampling from a system, addition of materials to a system, transfer of cultivated cells, and removal of material, products, and effluent from a system.	Minimize *Procedure* K-II-F	Minimize *Engineer* K-III-B K-III-D	Prevent *Engineer* K-IV-B K-IV-D	Prevent *Engineer* K-V-B K-V-D
11.	Treatment of exhaust gases from a closed system to minimize or prevent release of viable organisms.	NR	Minimize K-III-E	Prevent K-IV-E	Prevent K-V-E
12.	Closed system that has contained viable organisms not to be opened until sterilized by a validated procedure.	NR	K-III-F	K-IV-F	K-V-F
13.	Closed system to be maintained at as a low pressure as possible to maintain integrity of containment features.	NR	NR	NR	K-V-G
14.	Rotating seals and other penetrations into closed system designed to prevent or minimize leakage.	NR	NR	Prevent K-IV-G	Prevent K-V-H
15.	Closed system shall incorporate monitoring or sensing devices to monitor the integrity of containment.	NR	NR	K-IV-H	K-V-I
16.	Validated integrity testing of closed containment system.	NR	NR	K-IV-I	K-V-J
17.	Closed system to be permanently identified for record keeping purposes.	NR	NR	K-IV-J	K-V-K
18.	Universal biosafety sign to be posted on each closed system.	NR	NR	K-IV-K	K-V-L
19.	Emergency plans required for handling large losses of cultures.	K-II-G	K-III-G	K-IV-L	K-V-M
20.	Access to the work place.	NR	G-II-A-1-a	G-II-B-1-a	K-V-N
21.	Requirements for controlled access area.	NR	NR	NR	K-V-N&O

NR = not required

Appendix K-VI. Footnotes of Appendix K

Appendix K-VI-A. This table is derived from the text in Appendices G (*Physical Containment*) and K and is not to be used in lieu of Appendices G and K.

Appendix K-VI-B. The criteria in this grid address only the biological hazards associated with organisms containing recombinant or synthetic nucleic acid. Other hazards accompanying the large-scale cultivation of such organisms (e.g., toxic properties of products; physical, mechanical, and chemical aspects of downstream processing) are not addressed and shall be considered separately, albeit in conjunction with this grid.

Appendix K-VII. Definitions to Accompany Containment Grid and Appendix K

Appendix K-VII-A. Accidental Release. An accidental release is the unintentional discharge of a microbiological agent (i.e., microorganism or virus) or eukaryotic cell due to a failure in the containment system.

Appendix K-VII-B. Biological Barrier. A biological barrier is an impediment (naturally occurring or introduced) to the infectivity and/or survival of a microbiological agent or eukaryotic cell once it has been released into the environment.

Appendix K-VII-C. Closed System. A closed system is one in which by its design and proper operation, prevents release of a microbiological agent or eukaryotic cell contained therein.

Appendix K-VII-D. Containment. Containment is the confinement of a microbiological agent or eukaryotic cell that is being cultured, stored, manipulated, transported, or destroyed in order to prevent or limit its contact with people and/or the environment. Methods used to achieve this include: physical and biological barriers and inactivation using physical or chemical means.

Appendix K-VII-E. *De minimis* Release. *De minimis* release is the release of: (i) viable microbiological agents or eukaryotic cells that does not result in the establishment of disease in healthy people, plants, or animals; or (ii) in uncontrolled proliferation of any microbiological agents or eukaryotic cells.

Appendix K-VII-F. Disinfection. Disinfection is a process by which viable microbiological agents or eukaryotic cells are reduced to a level unlikely to produce disease in healthy people, plants, or animals.

Appendix K-VII-G. Good Large Scale Practice Organism. For an organism to qualify for Good Large Scale Practice consideration, it must meet the following criteria [Reference: Organization for Economic Cooperation and Development, *Recombinant DNA Safety Considerations*, 1987, p. 34-35]: (i) the host organism should be non-pathogenic, should not contain adventitious agents and should have an extended history of safe large-scale use or have built-in environmental limitations that permit optimum growth in the large-scale setting but limited survival without adverse consequences in the environment; (ii) the recombinant or synthetic nucleic acid molecule-engineered organism should be non-pathogenic, should be as safe in the large-scale setting as the host organism, and without adverse consequences in the environment; and (iii) the vector/insert should be well characterized and free from known harmful sequences; should be limited in size as much as possible to the DNA required to perform the intended function; should not increase the stability of the construct in the environment unless that is a requirement of the intended function; should be poorly mobilizable; and should not transfer any resistance markers to microorganisms unknown to acquire them naturally if such acquisition could compromise the use of a drug to control disease agents in human or veterinary medicine or agriculture.

Appendix K-VII-H. Inactivation. Inactivation is any process that destroys the ability of a specific microbiological agent or eukaryotic cell to self-replicate.

Appendix K-VII-I. Incidental Release. An incidental release is the discharge of a microbiological agent or eukaryotic cell from a containment system that is expected when the system is appropriately designed and properly operated and maintained.

Appendix K-VII-J. Minimization. Minimization is the design and operation of containment systems in order that any incidental release is a *de minimis* release.

Appendix K-VII-K. Pathogen. A pathogen is any microbiological agent or eukaryotic cell containing sufficient genetic information, which upon expression of such information, is capable of producing disease in healthy people, plants, or animals.

Appendix K-VII-L. Physical Barrier. A physical barrier is considered any equipment, facilities, or devices (e.g., fermentors, factories, filters, thermal oxidizers) which are designed to achieve containment.

Appendix K-VII-M. Release. Release is the discharge of a microbiological agent or eukaryotic cell from a containment system. Discharges can be incidental or accidental. Incidental releases are *de minimis* in nature; accidental releases may be *de minimis* in nature.

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APPENDIX L. GENE THERAPY POLICY CONFERENCES (GTPCS)

In order to enhance the depth and value of public discussion relevant to scientific, safety, social, and ethical implications of gene therapy research, the NIH Director will convene GTPCs at regular intervals. As appropriate, the NIH Director may convene a GTPC in conjunction with a RAC meeting. GTPCs will be administered by NIH/OBA. Conference participation will not involve a standing committee membership but rather will offer the unique advantage of assembling numerous participants who possess significant scientific, ethical, and legal expertise and/or interest that is directly applicable to a specific gene therapy research issue. At least one member of RAC will serve as Co-chair of each GTPC and report the findings of each GTPC to RAC at its next scheduled meeting. The RAC representative for each GTPC will be chosen based on the participant's area of expertise relative to the specific gene therapy research issue to be discussed. All RAC members will be invited to attend GTPCs. GTPCs will have representation from other Federal agencies, including FDA and OHRP. GTPCs will focus on broad overarching policy and scientific issues related to gene therapy research. Proposals for GTPC topics may be submitted by members of RAC, representatives of academia, industry, patient and consumer advocacy organizations, other Federal agencies, professional scientific societies, and the general public. GTPC topics will not be limited to discussion of human applications of gene therapy research, i.e., they may include basic research on the use of novel gene delivery vehicles, or novel applications of human gene transfer. The RAC, with the Director's approval, will have the primary responsibility for planning GTPC agendas. GTPC findings will be transmitted to the NIH Director and will be made publicly available. The NIH Director anticipates that this public policy forum will serve as a model for interagency communication and collaboration, concentrated expert discussion of novel scientific issues and their potential societal implications, and enhanced opportunity for public discussion of specific issues and potential impact of such applications on human health and the environment.

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APPENDIX M. POINTS TO CONSIDER IN THE DESIGN AND SUBMISSION OF PROTOCOLS FOR THE TRANSFER OF RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULES INTO ONE OR MORE HUMAN RESEARCH PARTICIPANTS (POINTS TO CONSIDER)

Appendix M applies to research conducted at or sponsored by an institution that receives any support for recombinant or synthetic nucleic acid molecule research from NIH. Researchers not covered by the *NIH Guidelines* are encouraged to use Appendix M (see Section I-C, *General Applicability*).

The acceptability of human somatic cell gene transfer has been addressed in several public documents as well as in numerous academic studies. In November 1982, the President's Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research published a report, *Splicing Life*, which resulted from a two-year process of public deliberation and hearings. Upon release of that report, a U.S. House of Representatives subcommittee held three days of public hearings with witnesses from a wide range of fields from the biomedical and social sciences to theology, philosophy, and law. In December 1984, the Office of Technology Assessment released a background paper, *Human Gene Therapy*, which concluded that civic, religious, scientific, and medical groups have all accepted, in principle, the appropriateness of gene transfer of somatic cells in humans for specific genetic diseases. Somatic cell gene transfer is seen as an extension of present methods that might be preferable to other technologies. In light of this public support, RAC is prepared to consider proposals for somatic cell gene transfer.

RAC will not at present entertain proposals for germ line alterations but will consider proposals involving somatic cell gene transfer. The purpose of somatic cell gene transfer is to treat an individual patient, e.g., by inserting a properly functioning gene into the subject's somatic cells. Germ line alteration involves a specific attempt to introduce genetic changes into the germ (reproductive) cells of an individual, with the aim of changing the set of genes passed on to the individual's offspring.

The RAC continues to explore the issues raised by the potential of *in utero* gene transfer clinical research. However, the RAC concludes that, at present, it is premature to undertake any *in utero* gene transfer clinical trial. Significant additional preclinical and clinical studies addressing vector transduction efficacy, biodistribution, and toxicity are required before a human *in utero* gene transfer protocol can proceed. In addition, a more thorough understanding of the development of human organ systems, such as the immune and nervous systems, is needed to better define the potential efficacy and risks of human *in utero* gene transfer. Prerequisites for considering any specific human *in utero* gene transfer procedure include an understanding of the pathophysiology of the candidate disease and a demonstrable advantage to the *in utero* approach. Once the above criteria are met, the RAC would be willing to consider well rationalized human *in utero* gene transfer clinical trials.

Research proposals involving the deliberate transfer of recombinant or synthetic nucleic acid molecules, or DNA or RNA derived from such nucleic acid molecules, into human subjects (human gene transfer) will be considered through a review process involving both NIH/OBA and RAC. Investigators shall submit their relevant information on the proposed human gene transfer experiments to NIH/OBA. Submission of human gene transfer protocols to NIH will be in the format described in Appendix M-I-A, *Submission Requirements for Protocol Submission.* Submission to NIH shall be for registration purposes and will ensure continued public access to relevant human gene transfer information conducted in compliance with the *NIH Guidelines*. Investigational New Drug (IND) applications should be submitted to FDA in the format described in 21 CFR, Chapter I, Subchapter D, Part 312, Subpart B, Section 23, *IND Content and Format*.

Institutional Biosafety Committee approval must be obtained from each institution at which recombinant or synthetic nucleic acid molecule material will be administered to human subjects (as opposed to each institution involved in the production of vectors for human application and each institution at which there is *ex vivo* transduction of recombinant or synthetic nucleic acid molecule material into target cells for human application).

Factors that may contribute to public discussion of a human gene transfer experiment by RAC include: (i) new vectors/new gene delivery systems, (ii) new diseases, (iii) unique applications of gene transfer, and (iv) other issues considered to require further public discussion. Among the experiments that may be considered exempt from RAC discussion are those determined not to represent possible risk to human health or the environment. Full, public RAC review and discussion of a human gene transfer experiment may be (1) initiated by the NIH Director; or (2) initiated by the NIH OBA Director following a recommendation to NIH OBA by: (a) three or more RAC members, or (b) a Federal agency other than NIH. An individual human gene transfer experiment that is recommended for full RAC review should represent novel characteristics deserving of public discussion. If it is determined that an experiment will undergo full RAC discussion, NIH/OBA will immediately notify the Principal Investigator. RAC members may forward individual requests for additional information relevant to a specific protocol through NIH/OBA to the Principal Investigator. In making a determination whether an experiment is novel, and thus deserving of full RAC discussion, reviewers will examine the scientific rationale, scientific context (relative to other proposals reviewed by RAC), whether the preliminary *in vitro* and *in vivo* safety data were obtained in appropriate models and are sufficient, and whether questions related to relevant social and ethical issues have been resolved. RAC recommendations on a specific human gene transfer experiment shall be forwarded to the NIH Director, the Principal Investigator, the sponsoring institution, and other DHHS components, as appropriate. Relevant documentation will be included in the material for the RAC meeting at which the experiment is scheduled to be discussed. RAC meetings will be open to the public except where trade secrets and proprietary information are reviewed (see Section IV-D-5, *Protection of Proprietary Data – Voluntary Compliance)*. RAC prefers that information provided in response to Appendix M contain no proprietary data or trade secrets, enabling all aspects of the review to be open to the public.

Note: Any application submitted to NIH/OBA shall not be designated as 'confidential' in its entirety. In the event that a sponsor determines that specific responses to one or more of the items described in Appendix M should be considered as proprietary or trade secret, each item should be clearly identified as such. The cover letter (attached to the submitted material) shall: (1) clearly indicate that select portions of the application contain information considered as proprietary or trade secret, (2) a brief explanation as to the reason that each of these items is determined proprietary or trade secret.

Public discussion of human gene transfer experiments (and access to relevant information) shall serve to inform the public about the technical aspects of the proposals, meaning and significance of the research, and significant safety, social, and ethical implications of the research. RAC discussion is intended to ensure safe and ethical conduct of gene transfer experiments and facilitate public understanding of this novel area of biomedical research.

In its evaluation of human gene transfer proposals, RAC will consider whether the design of such experiments offers adequate assurance that their consequences will not go beyond their purpose, which is the same as the traditional purpose of clinical investigation, namely, to protect the health and well being of human subjects being treated while at the same time gathering generalizable knowledge. Two possible undesirable consequences of the transfer of recombinant or synthetic nucleic acid molecules would be unintentional: (i) vertical transmission of genetic changes from an individual to his/her offspring, or (ii) horizontal transmission of viral infection to other persons with whom the individual comes in contact. Accordingly, Appendices M-I through M-V request information that will enable RAC and NIH/OBA to assess the possibility that the proposed experiment(s) will inadvertently affect reproductive cells or lead to infection of other people (e.g., medical personnel or relatives).

Appendix M will be considered for revisions as experience in evaluating proposals accumulates and as new scientific developments occur. This review will be carried out periodically as needed.

Appendix M-I. Requirements for Protocol Submission, Review, and Reporting – Human Gene Transfer Experiments

Appendix M-I-A. Requirements for Protocol Submission

The following documentation must be submitted (see exemption in Appendix M-VI-A, *Footnotes of Appendix M*) in printed or electronic form to the: Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax), E-mail: rosenthg@od.nih.gov. NIH OBA will confirm receipt within three working days after receiving the submission. Investigators should contact OBA if they do not receive this confirmation.

1. A cover letter on institutional letterhead, signed by the Principal Investigator(s), that: (1) acknowledges that the documentation submitted to NIH OBA complies with the requirements set forth in Appendix M-I-A, *Requirements for Protocol Submission*; (2) identifies the Institutional Biosafety Committee (IBC) and Institutional Review Board (IRB) at the proposed clinical trial site(s) responsible for local review and approval of the protocol; and (3) acknowledges that no research participant will be enrolled (see definition of enrollment in Section I-E-7) until the RAC review process has been completed (see Appendix M-I-B, *RAC Review Requirements*); IBC approval (from the clinical trial site) has been obtained; IRB approval has been obtained; and all applicable regulatory authorizations have been obtained.

2. The scientific abstract.

3. The non-technical abstract.

4. The proposed clinical protocol, including tables, figures, and relevant manuscripts.

5. Responses to Appendices M-II through M-V, *Description of the Proposal, Informed Consent, Privacy and Confidentiality, and Special Issues*. Responses to Appendices M-II through M-V may be provided either as an appendix to the clinical protocol or incorporated in the clinical protocol. If responses to Appendices M-II through M-V are incorporated in the clinical protocol, each response must refer to the appropriate Appendix M-II through M-V.

6. The proposed informed consent document (see Appendix M-III, *Informed Consent*).

7. Curriculum vitae of the Principal Investigator(s) (no more than two pages in biographical sketch format).

Note: A human gene transfer experiment submitted to NIH OBA should not contain confidential commercial information or trade secrets, enabling all aspects of the review to be open to the public.

Appendix M-I-B. RAC Review Requirements

Appendix M-I-B-1. Initial RAC Review

The initial RAC review process shall include a determination as to whether the human gene transfer experiment presents characteristics that warrant public RAC review and discussion. During the RAC's initial review, individual committee members may request additional information relevant to the protocol. NIH OBA will immediately notify the Principal Investigator(s) of RAC requests for additional information. In making a determination whether an experiment presents characteristics warranting public RAC review and discussion, reviewers will examine the scientific rationale, scientific content, whether the preliminary *in vitro* and *in vivo* safety data were obtained in appropriate models and are sufficient, and whether questions related to relevant social and ethical issues have been resolved. Other factors that may warrant public review and discussion of a human gene transfer experiment by the RAC include: (1) a new vector/new gene delivery system; (2) a new clinical application; (3) a unique application of gene transfer; and/or (4) other issues considered to require further public discussion.

Initial RAC review shall be completed within 15 working days of receipt of a complete submission (see Appendix M-I-A, *Requirements for Protocol Submission*). At the end of the 15-day review period, NIH OBA will notify the Principal Investigator(s) in writing about the results of the RAC's initial review. Two outcomes are possible: (1) the experiment does not present characteristics that warrant further review and discussion and is therefore exempt from public RAC review and discussion; or (2) the experiment presents characteristics that warrant public RAC review and discussion. Completion of the RAC review process is defined as: (1) receipt by the Principal Investigator(s) of a letter from NIH OBA indicating that the submission does not present characteristics that warrant public RAC review and discussion; or (2) receipt by the Principal Investigator(s) of a letter from NIH OBA after public RAC review that summarizes the committee's key comments and recommendations (if any).

If a human gene transfer protocol is submitted less than eight weeks before a scheduled RAC meeting and is subsequently recommended for public RAC review and discussion, the review of the protocol by the RAC will be deferred until the next scheduled RAC meeting. This eight-week period is needed to ensure adequate time for public notice and comment and thorough review by the committee members.

No research participant shall be enrolled (see definition of enrollment in Section I-E-7) in the human gene transfer experiment until: (1) the RAC review process has been completed; (2) Institutional Biosafety Committee (IBC) approval (from the clinical trial site) has been obtained; (3) Institutional Review Board (IRB) approval has been obtained; and (4) all applicable regulatory authorization(s) have been obtained.

For a clinical trial site that is added after the RAC review process, no research participant shall be enrolled (see definition of enrollment in Section I-E-7) at the clinical trial site until the following documentation has been submitted to NIH OBA: (1) IBC approval (from the clinical trial site); (2) IRB approval; (3) IRB-approved informed consent document; (4) curriculum vitae of the Principal Investigator(s) (no more than two pages in biographical sketch format); and (5) NIH grant numbers(s) if applicable.

Appendix M-I-B-2. Public RAC Review and Discussion

Public RAC review and discussion of a human gene transfer experiment may be: (1) initiated by the NIH Director; or (2) initiated by the NIH OBA Director following a recommendation to NIH OBA by: (a) three or more RAC members; or (b) a Federal agency other than NIH. In making a determination whether an experiment presents characteristics warranting public RAC review and discussion, reviewers will examine the scientific rationale, scientific content, whether the preliminary *in vitro* and *in vivo* safety data were obtained in appropriate models and are sufficient, and whether questions related to relevant social and ethical issues have been

resolved. Other factors that may warrant public review and discussion of a human gene transfer experiment by the RAC include: (1) a new vector/new gene delivery system; (2) a new clinical application; (3) a unique application of gene transfer; and/or (4) other issues considered to require further public discussion.

After a human gene transfer experiment is reviewed by the full RAC at a regularly scheduled meeting, NIH OBA will send a letter summarizing the RAC key comments and recommendations (if any) regarding the protocol to the NIH Director, the Principal Investigator, the sponsoring institution, and other DHHS components, as appropriate. Completion of RAC review is defined as receipt by the Principal Investigator(s) of a letter from NIH OBA summarizing the committee's findings. Unless NIH OBA determines that there are exceptional

circumstances, the RAC summary letter will be sent to the Principal Investigator(s) within 10 working days after the completion of the RAC meeting at which the experiment was reviewed.

RAC meetings will be open to the public except where trade secrets or confidential commercial information are reviewed. To enable all aspects of the protocol review process to be open to the public, information provided in response to Appendix M should not contain trade secrets or confidential commercial information. No application submitted to NIH OBA shall be designated as 'confidential' in its entirety. In the event that an investigator determines that specific responses to one or more of the items described in Appendix M should be considered as confidential commercial information or a trade secret, each item must be clearly identified as such. The cover letter (attached to the submitted material) shall: (1) clearly designate the information that is considered as confidential commercial information or a trade secret; and (2) explain and justify each designation.

Appendix M-I-C. Reporting Requirements

Appendix M-I-C-1. Initiation of the Clinical Investigation

No later than 20 working days after enrollment (see definition of enrollment in Section I-E-7) of the first research participant in a human gene transfer experiment, the Principal Investigator(s) shall submit the following documentation to NIH OBA: (1) a copy of the informed consent document approved by the Institutional Review Board (IRB); (2) a copy of the protocol approved by the Institutional Biosafety Committee (IBC) and IRB; (3) a copy of the final IBC approval from the clinical trial site; (4) a copy of the final IRB approval; (5) a brief written report that includes the following information: (a) how the investigator(s) responded to each of the RAC's recommendations on the protocol (if applicable); and (b) any modifications to the protocol as required by FDA; (6) applicable NIH grant number(s); (7) the FDA Investigational New Drug Application (IND) number; and (8) the date of the initiation of the trial. The purpose of requesting the FDA IND number is for facilitating interagency collaboration in the Federal oversight of human gene transfer research.

Appendix M-I-C-2. Additional Clinical Trial Sites

No research participant shall be enrolled (see definition of enrollment in Section I-E-7) at a clinical trial site until the following documentation has been submitted to NIH OBA: (1) Institutional Biosafety Committee approval (from the clinical trial site); (2) Institutional Review Board approval; (3) Institutional Review Board-approved informed consent document; (4) curriculum vitae of the Principal Investigator(s) (no more than two pages in biographical sketch format); and (5) NIH grant number(s) if applicable.

Appendix M-I-C-3. Annual Reports

Within 60 days after the one-year anniversary of the date on which the investigational new drug (IND) application went into effect, and after each subsequent anniversary until the trial is completed, the Principal Investigator (or delegate) shall submit the information set forth in (a), (b), and (c). When multiple studies are conducted under the single IND, the Principal Investigator (or delegate) may choose to submit a single annual report covering all studies, provided that each study is identified by its OBA protocol number.

(a) Clinical Trial Information. A brief summary of the status of each trial in progress and each trial completed during the previous year. The summary is required to include the following information for each trial: (1) the title and purpose of the trial; (2) clinical site; (3) the Principal Investigator; (4) clinical protocol identifiers, including the NIH OBA protocol number, NIH grant number(s) (if applicable), and the FDA IND application number; (5) participant population (such as disease indication and general age group, e.g., adult or pediatric); (6) the total number of participants planned for inclusion in the trial; the number entered into the trial to date; the number

whose participation in the trial was completed; and the number who dropped out of the trial with a brief description of the reasons; (7) the status of the trial, e.g., open to accrual of subjects, closed but data collection ongoing, or fully completed, and (8) if the trial has been completed, a brief description of any study results.

(b) Progress Report and Data Analysis. Information obtained during the previous year's clinical and non-clinical investigations, including: (1) a narrative or tabular summary showing the most frequent and most serious adverse experiences by body system; (2) a summary of all serious adverse events submitted during the past year; (3) a summary of serious adverse events that were expected or considered to have causes not associated with the use of the gene transfer product such as disease progression or concurrent medications; (4) if any deaths have occurred, the number of participants who died during participation in the investigation and causes

of death; and (5) a brief description of any information obtained that is pertinent to an understanding of the gene transfer product's actions, including, for example, information about dose-response, information from controlled trials, and information about bioavailability.

(c) A copy of the updated clinical protocol including a technical and non-technical abstract.

Appendix M-I-C-4. Safety Reporting

Principal Investigators must submit, in accordance with this section, Appendix M-I-C-4-a and Appendix M-I-C-4-b, a written report on: (1) any serious adverse event that is both unexpected and associated with the use of the gene transfer product (i.e., there is reasonable possibility that the event may have been caused by the use of the product; investigators should not await definitive proof of association before reporting such events); and (2) any finding from tests in laboratory animals that suggests a significant risk for human research participants including reports of mutagenicity, teratogenicity, or carcinogenicity. The report must be clearly labeled as a "Safety Report" and must be submitted to the NIH Office of Biotechnology Activities (NIH OBA) and to the local Institutional Biosafety Committee within the timeframes set forth in Appendix M-I-C-4-b.

Principal Investigators should adhere to any other serious adverse event reporting requirements in accordance with federal regulations, state laws, and local institutional policies and procedures, as applicable.

Principal Investigators may delegate to another party, such as a corporate sponsor, the reporting functions set forth in Appendix M, with written notification to the NIH OBA of the delegation and of the name(s), address, telephone and fax numbers of the contact(s). The Principal Investigator is responsible for ensuring that the reporting requirements are fulfilled and will be held accountable for any reporting lapses.

The three alternative mechanisms for reporting serious adverse events to the NIH OBA are: by e-mail to oba@od.nih.gov; by fax to 301-496-9839; or by mail to the Office of Biotechnology Activities, National Institutes of Health, MSC 7985, 6705 Rockledge Drive, Suite 750, Bethesda, Maryland 20892-7985.

Appendix M-I-C-4-a. Safety Reporting: Content and Format

The serious adverse event report must include, but need not be limited to: (1) the date of the event; (2) designation of the report as an initial report or a follow-up report, identification of all safety reports previously filed for the clinical protocol concerning a similar adverse event, and an analysis of the significance of the adverse event in light of previous similar reports; (3) clinical site; (4) the Principal Investigator; (5) NIH Protocol number; (6) FDA's Investigational New Drug (IND) Application number; (7) vector type , e.g., adenovirus; (8) vector subtype, e.g., type 5, relevant deletions; (9) gene delivery method, e.g., *in vivo, ex vivo* transduction; (10) route of administration, e.g., intratumoral, intravenous; (11) dosing schedule; (12) a complete description of the event; (13) relevant clinical observations; (14) relevant clinical history; (15) relevant tests that were or are planned to be conducted; (16) date of any treatment of the event; and (17) the suspected cause of the event. These items may be reported by using the recommended Adverse Event Reporting Template available on NIH OBA's web site at: http://oba.od.nih.gov/rdna/adverse_event_oba.html, the FDA MedWatch forms, or other means provided that all of the above elements are specifically included.

Reports from laboratory animal studies as delineated in Appendix M-I-C-4 must be submitted in a narrative format.

Appendix M-I-C-4-b. Safety Reporting: Time frames for Expedited Reports

Any serious adverse event that is fatal or life-threatening, that is unexpected, and associated with the use of the gene transfer product must be reported to the NIH OBA as soon as possible, but not later than 7 calendar days after the sponsor's initial receipt of the information (i.e., at the same time the event must be reported to the FDA).

Serious adverse events that are unexpected and associated with the use of the gene transfer product, but are not fatal or life-threatening, must be reported to the NIH OBA as soon as possible, but not later than 15 calendar days after the sponsor's initial receipt of the information (i.e., at the same time the event must be reported to the FDA).

Changes in this schedule are permitted only where, under the FDA IND regulations [21 CFR 312(c)(3)], changes

in this reporting schedule have been approved by the FDA and are reflected in the protocol.

If, after further evaluation, an adverse event initially considered not to be associated with the use of the gene transfer product is subsequently determined to be associated, then the event must be reported to the NIH OBA within 15 days of the determination.

Relevant additional clinical and laboratory data may become available following the initial serious adverse event report. Any follow-up information relevant to a serious adverse event must be reported within 15 calendar days of the sponsor's receipt of the information. If a serious adverse event occurs after the end of a clinical trial and is determined to be associated with the use of the gene transfer product, that event shall be reported to the NIH OBA within 15 calendar days of the determination.

Any finding from tests in laboratory animals that suggests a significant risk for human research participants including reports of mutagenicity, teratogenicity, or carcinogenicity must be reported as soon as possible, but not later than 15 calendar days after the sponsor's initial receipt of the information (i.e., at the same time the event must be reported to the FDA).

Appendix M-I-C-5. Confidentiality

Data submitted in accordance with Appendix M-I-C that are claimed to be confidential commercial or trade secret information must be clearly labeled as such. Prior to making its determination about the confidentiality of data labeled confidential commercial or trade secret, the NIH will contact the Principal Investigator or delegate to ascertain the basis for the claim and subsequently will notify the Principal Investigator or delegate of its final determination regarding the claim.

If NIH determines that the data so labeled are confidential commercial or trade secret and that their public disclosure would promote an understanding of key scientific or safety issues, the NIH will seek agreement from the appropriate party to release such data. Public discussion of scientific and safety issues raised by data submitted in accordance with Appendix M-I-C is vital to informing both investigators and human subjects about the safety of gene transfer research.

To protect the privacy of participants in gene transfer research, any serious adverse event or annual reports submitted to NIH OBA must not contain any information that would identify the human research participants.

Appendix M-I-D. Safety Assessment in Human Gene Transfer Research

A working group of the RAC, the NIH Gene Transfer Safety Assessment Board, with staff support from the NIH OBA, will: 1) review in closed session as appropriate safety information from gene transfer trials for the purpose of assessing toxicity and safety data across gene transfer trials; 2) identify significant trends or significant single events; and 3) report significant findings and aggregated trend data to the RAC. It is expected that this process will enhance review of new protocols, improve the development, design, and conduct of human gene transfer trials, promote public understanding and awareness of the safety of human gene transfer research studies, and inform the decision-making of potential trial participants.

Appendix M-II. Description of the Proposal

Responses to this appendix should be provided in the form of either written answers or references to specific sections of the protocol or its appendices. Investigators should indicate the points that are not applicable with a brief explanation. Investigators submitting proposals that employ the same vector systems may refer to preceding documents relating to the vector sequence without having to rewrite such material.

Appendix M-II-A. Objectives and Rationale of the Proposed Research

State concisely the overall objectives and rationale of the proposed study. Provide information on the specific points that relate to whichever type of research is being proposed.

Appendix M-II-A-1. Use of Recombinant or Synthetic Nucleic Acid Molecules for Therapeutic Purposes

For research in which recombinant or synthetic nucleic acid molecules are transferred in order to treat a disease or disorder (e.g., genetic diseases, cancer, and metabolic diseases), the following questions should be addressed:

Appendix M-II-A-1-a. Why is the disease selected for experimental treatment by means of gene transfer a good candidate for such treatment?

Appendix M-II-A-1-b. Describe the natural history and range of expression of the disease selected for experimental treatment. What objective and/or quantitative measures of disease activity are available? In your view, are the usual effects of the disease predictable enough to allow for meaningful assessment of the results of gene transfer?

Appendix M-II-A-1-c. Is the protocol designed to prevent all manifestations of the disease, to halt the progression of the disease after symptoms have begun to appear, or to reverse manifestations of the disease in seriously ill victims?

Appendix M-II-A-1-d. What alternative therapies exist? In what groups of subjects are these therapies effective? What are their relative advantages and disadvantages as compared with the proposed gene transfer?

Appendix M-II-A-2. Transfer of Recombinant or Synthetic Nucleic Acid Molecules for Other Purposes

Appendix M-II-A-2-a. Into what cells will the recombinant or synthetic nucleic acid molecules be transferred? Why is the transfer of recombinant or synthetic nucleic acid molecules necessary for the proposed research? What questions can be answered by using recombinant or synthetic nucleic acid molecules?

Appendix M-II-A-2-b. What alternative methodologies exist? What are their relative advantages and disadvantages as compared to the use of recombinant or synthetic nucleic acid molecules?

Appendix M-II-B. Research Design, Anticipated Risks and Benefits

Appendix M-II-B-1. Structure and Characteristics of the Biological System

Provide a full description of the methods and reagents to be employed for gene delivery and the rationale for their use. The following are specific points to be addressed:

Appendix M-II-B-1-a. What is the structure of the cloned DNA that will be used?

Appendix M-II-B-1-a-(1). Describe the gene (genomic or cDNA), the bacterial plasmid or phage vector, and the delivery vector (if any). Provide complete nucleotide sequence analysis or a detailed restriction enzyme map of the total construct.

Appendix M-II-B-1-a-(2). What regulatory elements does the construct contain (e.g., promoters, enhancers, polyadenylation sites, replication origins, etc.)? From what source are these elements derived? Summarize what is currently known about the regulatory character of each element.

Appendix M-II-B-1-a-(3). Describe the steps used to derive the recombinant or synthetic nucleic acid construct.

Appendix M-II-B-1-b. What is the structure of the material that will be administered to the research participant?

Appendix M-II-B-1-b-(1). Describe the preparation, structure, and composition of the materials that will be given to the human research subject or used to treat the subject's cells: (i) If recombinant or synthetic nucleic acid, what is the purity (both in terms of being a single nucleic acid species and in terms of other contaminants)? What tests have been used and what is the sensitivity of the tests? (ii) If a virus, how is it prepared from the recombinant or synthetic nucleic acid construct? In what cell is the virus grown (any special features)? What medium and serum are used? How is the virus purified? What is its structure and purity? What steps are being taken (and assays used with their sensitivity) to detect and eliminate any contaminating materials (for example,

VL30 RNA, other nucleic acids, or proteins) or contaminating viruses (both replication-competent or replication-defective) or other organisms in the cells or serum used for preparation of the virus stock including any contaminants that may have biological effects? (iii) If co-cultivation is employed, what kinds of cells are being used for co-cultivation? What steps are being taken (and assays used with their sensitivity) to detect and eliminate any contaminating materials? Specifically, what tests are being conducted to assess the material to be returned to the subject for the presence of live or killed donor cells or other non-vector materials (for example, VL30 sequences) originating from those cells? (iv) If methods other than those covered by Appendices M-II-B-1 through M-II-B-3, *Research Design, Anticipated Risks and Benefits*, are used to introduce new genetic information into target cells, what steps are being taken to detect and eliminate any contaminating materials? What are possible sources of contamination? What is the sensitivity of tests used to monitor contamination?

Appendix M-II-B-1-b-(2). Describe any other material to be used in preparation of the material to be administered to the human research subject. For example, if a viral vector is proposed, what is the nature of the helper virus or cell line? If carrier particles are to be used, what is the nature of these?

Appendix M-II-B-2. Preclinical Studies, Including Risk-Assessment Studies

Provide results that demonstrate the safety, efficacy, and feasibility of the proposed procedures using animal and/or cell culture model systems, and explain why the model(s) chosen is/are most appropriate.

Appendix M-II-B-2-a. Delivery System

Appendix M-II-B-2-a-(1). What cells are the intended target cells of the recombinant or synthetic nucleic acid molecules? What target cells are to be treated *ex vivo* and returned to the human subject, how will the cells be characterized before and after treatment? What is the theoretical and practical basis for assuming that only the target cells will incorporate the recombinant or synthetic nucleic acid?

Appendix M-II-B-2-a-(2). Is the delivery system efficient? What percentage of the target cells contain the added recombinant or synthetic nucleic acid?

Appendix M-II-B-2-a-(3). How is the structure of the added recombinant or synthetic nucleic acid sequences monitored and what is the sensitivity of the analysis? Is the added DNA extrachromosomal or integrated? Is the added recombinant or synthetic nucleic acid unrearranged?

Appendix M-II-B-2-a-(4). How many copies are present per cell? How stable is the added recombinant or synthetic nucleic acid both in terms of its continued presence and its structural stability?

Appendix M-II-B-2-b. Gene Transfer and Expression

Appendix M-II-B-2-b-(1). What animal and cultured cell models were used in laboratory studies to assess the *in vivo* and *in vitro* efficacy of the gene transfer system? In what ways are these models similar to and different from the proposed human treatment?

Appendix M-II-B-2-b-(2). What is the minimal level of gene transfer and/or expression that is estimated to be necessary for the gene transfer protocol to be successful in humans? How was this level determined?

Appendix M-II-B-2-b-(3). Explain in detail all results from animal and cultured cell model experiments which assess the effectiveness of the delivery system in achieving the minimally required level of gene transfer and expression.

Appendix M-II-B-2-b-(4). To what extent is expression only from the desired gene (and not from the surrounding DNA)? To what extent does the insertion modify the expression of other genes?

Appendix M-II-B-2-b-(5). In what percentage of cells does expression from the added recombinant or synthetic nucleic acid occur? Is the product biologically active? What percentage of normal activity results from the inserted gene?

Appendix M-II-B-2-b-(6). Is the gene expressed in cells other than the target cells? If so, to what extent?

Appendix M-II-B-2-c. Retrovirus Delivery Systems

Appendix M-II-B-2-c-(1). What cell types have been infected with the retroviral vector preparation? Which cells, if any, produce infectious particles?

Appendix M-II-B-2-c-(2). How stable are the retroviral vector and the resulting provirus against loss, rearrangement, recombination, or mutation? What information is available on how much rearrangement or recombination with endogenous or other viral sequences is likely to occur in the human subject's cells? What steps have been taken in designing the vector to minimize instability or variation? What laboratory studies have been performed to check for stability, and what is the sensitivity of the analyses?

Appendix M-II-B-2-c-(3). What laboratory evidence is available concerning potential harmful effects of the transfer (e.g., development of neoplasia, harmful mutations, regeneration of infectious particles, or immune responses)? What steps will be taken in designing the vector to minimize pathogenicity? What laboratory studies have been performed to check for pathogenicity, and what is the sensitivity of the analyses?

Appendix M-II-B-2-c-(4). Is there evidence from animal studies that recombinant or synthetic vector nucleic acid has entered untreated cells, particularly germ-line cells? What is the sensitivity of these analyses?

Appendix M-II-B-2-c-(5). Has a protocol similar to the one proposed for a clinical trial been conducted in non-human primates and/or other animals? What were the results? Specifically, is there any evidence that the retroviral vector has recombined with any endogenous or other viral sequences in the animals?

Appendix M-II-B-2-d. Non-Retrovirus Delivery/Expression Systems

If a non-retroviral delivery system is used, what animal studies have been conducted to determine if there are pathological or other undesirable consequences of the protocol (including insertion of DNA into cells other than those treated, particularly germ-line cells)? How long have the animals been studied after treatment? What safety studies have been conducted? (Include data about the level of sensitivity of such assays.)

Appendix M-II-B-3. Clinical Procedures, Including Research Participant Monitoring

Describe the experimental treatment that will be administered to the human subjects and the diagnostic methods that will be used to monitor the success or failure of the experimental treatment. If previous clinical studies using similar methods have been performed by yourself or others, indicate their relevance to the proposed study. Specifically:

Appendix M-II-B-3-a. Will cells (e.g., bone marrow cells) be removed from human subjects and treated *ex vivo*? If so, describe the type, number, and intervals at which these cells will be removed.

Appendix M-II-B-3-b. Will human subjects be treated to eliminate or reduce the number of cells containing malfunctioning genes (e.g., through radiation or chemotherapy)?

Appendix M-II-B-3-c. What treated cells (or vector/nucleic acid combination) will be given to human subjects? How will the treated cells be administered? What volume of cells will be used? Will there be single or multiple experimental treatments? If so, over what period of time?

Appendix M-II-B-3-d. How will it be determined that new gene sequences have been inserted into the subject's cells and if these sequences are being expressed? Are these cells limited to the intended target cell populations? How sensitive are these analyses?

Appendix M-II-B-3-e. What studies will be conducted to assess the presence and effects of the contaminants?

Appendix M-II-B-3-f. What are the clinical endpoints of the study? Are there objectives and quantitative measurements to assess the natural history of the disease? Will such measurements be used in human subject follow-up? How will subjects be monitored to assess specific effects of the treatment on the disease? What is the sensitivity of the analyses? How frequently will follow-up studies be conducted? How long will follow-up continue?

Appendix M-II-B-3-g. What are the major beneficial and adverse effects of the experimental treatment that you anticipate? What measures will be taken in an attempt to control or reverse these adverse effects if they occur? Compare the probability and magnitude of deleterious consequences from the disease if nucleic acid molecule transfer is not used.

Appendix M-II-B-3-h. If a treated human subject dies, what special post-mortem studies will be performed?

Appendix M-II-B-4. Public Health Considerations

Describe any potential benefits and hazards of the proposed gene transfer to persons other than the human subjects receiving the experimental treatment. Specifically:

Appendix M-II-B-4-a. On what basis are potential public health benefits or hazards postulated?

Appendix M-II-B-4-b. Is there a significant possibility that the added recombinant or synthetic nucleic acid will spread from the human subject to other persons or to the environment?

Appendix M-II-B-4-c. What precautions will be taken against such spread (e.g., subjects sharing a room, health-care workers, or family members)?

Appendix M-II-B-4-d. What measures will be undertaken to mitigate the risks, if any, to public health?

Appendix M-II-B-4-e. In light of possible risks to offspring, including vertical transmission, will birth control measures be recommended to subjects? Are such concerns applicable to health care personnel?

Appendix M-II-B-5. Qualifications of Investigators and Adequacy of Laboratory and Clinical Facilities

Indicate the relevant training and experience of the personnel who will be involved in the preclinical studies and clinical administration of recombinant or synthetic nucleic acid molecules. Describe the laboratory and clinical facilities where the proposed study will be performed. Specifically:

Appendix M-II-B-5-a. What professional personnel (medical and nonmedical) will be involved in the proposed study and what is their relevant expertise? Provide a two-page curriculum vitae for each key professional person in biographical sketch format (see Appendix M-I-A, *Requirements for Protocol Submission*).

Appendix M-II-B-5-b. At what hospital or clinic will the experimental treatment be given? Which facilities of the hospital or clinic will be especially important for the proposed study? Will subjects occupy regular hospital beds or clinical research center beds? Where will subjects reside during the follow-up period? What special arrangements will be made for the comfort and consideration of the research participants. Will the research institution designate an ombudsman, patient care representative, or other individual to help protect the rights and welfare of the research participant?

Appendix M-II-C. Selection of the Human Subjects

Estimate the number of human subjects to be involved in the proposed study. Describe recruitment procedures and eligibility requirements, paying particular attention to whether these procedures and requirements are fair and equitable. Specifically:

Appendix M-II-C-1. How many subjects do you plan to involve in the proposed study?

Appendix M-II-C-2. How many eligible subjects do you anticipate being able to identify each year?

Appendix M-II-C-3. What recruitment procedures do you plan to use?

Appendix M-II-C-4. What selection criteria do you plan to employ? What are the exclusion and inclusion criteria for the study?

Appendix M-II-C-5. How will subjects be selected if it is not possible to include all who desire to participate?

Appendix M-III. Informed Consent

In accordance with the Protection of Human Subjects (45 CFR Part 46), investigators should indicate how subjects will be informed about the proposed study and the manner in which their consent will be solicited. They should indicate how the Informed Consent document makes clear the special requirements of gene transfer research. If a proposal involves children, special attention should be paid to the Protection of Human Subjects (45 CFR Part 46), Subpart D, Additional Protections for Children Involved as Subjects in Research.

Appendix M-III-A. Communication About the Study to Potential Participants

Appendix M-III-A-1. Which members of the research group and/or institution will be responsible for contacting potential participants and for describing the study to them? What procedures will be used to avoid possible conflicts of interest if the investigator is also providing medical care to potential subjects?

Appendix M-III-A-2. How will the major points covered in Appendix M-II, *Description of Proposal*, be disclosed to potential participants and/or their parents or guardians in language that is understandable to them?

Appendix M-III-A-3. What is the length of time that potential participants will have to make a decision about their participation in the study?

Appendix M-III-A-4. If the study involves pediatric or mentally handicapped subjects, how will the assent of each person be obtained?

Appendix M-III-B. Informed Consent Document

Submission of a human gene transfer experiment to NIH OBA must include a copy of the proposed informed consent document. A separate Informed Consent document should be used for the gene transfer portion of a research project when gene transfer is used as an adjunct in the study of another technique, e.g., when a gene is used as a "marker" or to enhance the power of immunotherapy for cancer.

Because of the relative novelty of the procedures that are used, the potentially irreversible consequences of the procedures performed, and the fact that many of the potential risks remain undefined, the Informed Consent document should include the following specific information in addition to any requirements of the DHHS regulations for the Protection of Human Subjects (45 CFR 46). Indicate if each of the specified items appears in the Informed Consent document or, if not included in the Informed Consent document, how those items will be presented to potential subjects. Include an explanation if any of the following items are omitted from the consent process or the Informed Consent document.

Appendix M-III-B-1. General Requirements of Human Subjects Research

Appendix M-III-B-1-a. Description/Purpose of the Study

The subjects should be provided with a detailed explanation in non-technical language of the purpose of the study and the procedures associated with the conduct of the proposed study, including a description of the gene transfer component.

Appendix M-III-B-1-b. Alternatives

The Informed Consent document should indicate the availability of therapies and the possibility of other investigational interventions and approaches.

Appendix M-III-B-1-c. Voluntary Participation

The subjects should be informed that participation in the study is voluntary and that failure to participate in the study or withdrawal of consent will not result in any penalty or loss of benefits to which the subjects are otherwise entitled.

Appendix M-III-B-1-d. Benefits

The subjects should be provided with an accurate description of the possible benefits, if any, of participating in the proposed study. For studies that are not reasonably expected to provide a therapeutic benefit to subjects, the Informed Consent document should clearly state that no direct clinical benefit to subjects is expected to occur as a result of participation in the study, although knowledge may be gained that may benefit others.

Appendix M-III-B-1-e. Possible Risks, Discomforts, and Side Effects

There should be clear itemization in the Informed Consent document of types of adverse experiences, their relative severity, and their expected frequencies. For consistency, the following definitions are suggested: side effects that are listed as mild should be ones which do not require a therapeutic intervention; moderate side effects require an intervention; and severe side effects are potentially fatal or life-threatening, disabling, or require prolonged hospitalization.

If verbal descriptors (e.g., "rare," "uncommon," or "frequent") are used to express quantitative information regarding risk, these terms should be explained.

The Informed Consent document should provide information regarding the approximate number of people who have previously received the genetic material under study. It is necessary to warn potential subjects that, for genetic materials previously used in relatively few or no humans, unforeseen risks are possible, including ones that could be severe.

The Informed Consent document should indicate any possible adverse medical consequences that may occur if the subjects withdraw from the study once the study has started.

Appendix M-III-B-1-f. Costs

The subjects should be provided with specific information about any financial costs associated with their participation in the protocol and in the long-term follow-up to the protocol that are not covered by the investigators or the institution involved.

Subjects should be provided an explanation about the extent to which they will be responsible for any costs for medical treatment required as a result of research-related injury.

Appendix M-III-B-2. Specific Requirements of Gene Transfer Research

Appendix M-III-B-2-a. Reproductive Considerations

To avoid the possibility that any of the reagents employed in the gene transfer research could cause harm to a fetus/child, subjects should be given information concerning possible risks and the need for contraception by males and females during the active phase of the study. The period of time for the use of contraception should be specified.

The inclusion of pregnant or lactating women should be addressed.

Appendix M-III-B-2-b. Long-Term Follow-Up

To permit evaluation of long-term safety and efficacy of gene transfer, the prospective subjects should be informed that they are expected to cooperate in long-term follow-up that extends beyond the active phase of the study. The Informed Consent document should include a list of persons who can be contacted in the event that questions arise during the follow-up period. The investigator should request that subjects continue to provide a current address and telephone number.

The subjects should be informed that any significant findings resulting from the study will be made known in a timely manner to them and/or their parent or guardian including new information about the experimental procedure, the harms and benefits experienced by other individuals involved in the study, and any long-term effects that have been observed.

Appendix M-III-B-2-c. Request for Autopsy

To obtain vital information about the safety and efficacy of gene transfer, subjects should be informed that at the time of death, no matter what the cause, permission for an autopsy will be requested of their families. Subjects should be asked to advise their families of the request and of its scientific and medical importance.

Appendix M-III-B-2-d. Interest of the Media and Others in the Research

To alert subjects that others may have an interest in the innovative character of the protocol and in the status of the treated subjects, the subjects should be informed of the following: (i) that the institution and investigators will make efforts to provide protection from the media in an effort to protect the participants' privacy, and (ii) that representatives of applicable Federal agencies (e.g., the National Institutes of Health and the Food and Drug Administration), representatives of collaborating institutions, vector suppliers, etc., will have access to the subjects' medical records.

Appendix M-IV. Privacy

Indicate what measures will be taken to protect the privacy of subjects and their families as well as maintain the confidentiality of research data. These measures should help protect the confidentiality of information that could directly or indirectly identify study participants.

Appendix M-IV-A. What provisions will be made to honor the wishes of individual human subjects (and the parents or guardians of pediatric or mentally handicapped subjects) as to whether, when, or how the identity of a subject is publicly disclosed.

Appendix M-IV-B. What provisions will be made to maintain the confidentiality of research data, at least in cases where data could be linked to individual subjects?

Appendix M-V. Special Issues

Although the following issues are beyond the normal purview of local Institutional Review Boards, investigators should respond to the following questions:

Appendix M-V-A. What steps will be taken, consistent with Appendix M-IV, *Privacy*, to ensure that accurate and appropriate information is made available to the public with respect to such public concerns as may arise from the proposed study?

Appendix M-V-B. Do you or your funding sources intend to protect under patent or trade secret laws either the products or the procedures developed in the proposed study? If so, what steps will be taken to permit as full communication as possible among investigators and clinicians concerning research methods and results?

Appendix M-VI. Footnotes of Appendix M

Appendix M-VI-A. Human studies in which induction or enhancement of an immune response to a vector-encoded microbial immunogen is the major goal, such an immune response has been demonstrated in model systems, and the persistence of the vector-encoded immunogen is not expected, are exempt from Appendix M-I, *Requirements for Protocol Submission, Review and Reporting – Human Gene Transfer Experiments*.

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APPENDIX P. PHYSICAL AND BIOLOGICAL CONTAINMENT FOR RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULE RESEARCH INVOLVING PLANTS

Appendix P specifies physical and biological containment conditions and practices suitable to the greenhouse conduct of experiments involving recombinant or synthetic nucleic acid molecule-containing plants, plant-associated microorganisms, and small animals. All provisions of the *NIH Guidelines* apply to plant research activities with the following modifications:

Appendix P shall supersede Appendix G (*Physical Containment*) when the research plants are of a size, number, or have growth requirements that preclude the use of containment conditions described in Appendix G. The plants covered in Appendix P include but are not limited to mosses, liverworts, macroscopic algae, and vascular plants including terrestrial crops, forest, and ornamental species.

Plant-associated microorganisms include viroids, virusoids, viruses, bacteria, fungi, protozoans, certain small algae, and microorganisms that have a benign or beneficial association with plants, such as certain *Rhizobium* species and microorganisms known to cause plant diseases. The appendix applies to microorganisms which are being modified with the objective of fostering an association with plants.

Plant-associated small animals include those arthropods that: (i) are in obligate association with plants, (ii) are plant pests, (iii) are plant pollinators, or (iv) transmit plant disease agents, as well as other small animals such as nematodes for which tests of biological properties necessitate the use of plants. Microorganisms associated with such small animals (e.g., pathogens or symbionts) are included.

The Institutional Biosafety Committee shall include at least one individual with expertise in plant, plant pathogen, or plant pest containment principles when experiments utilizing Appendix P require prior approval by the Institutional Biosafety Committee.

Appendix P-I. General Plant Biosafety Levels

Appendix P-I-A. The principal purpose of plant containment is to avoid the unintentional transmission of a recombinant or synthetic nucleic acid molecule-containing plant genome, including nuclear or organelle hereditary material or release of recombinant or synthetic nucleic acid molecule-derived organisms associated with plants.

Appendix P-I-B. The containment principles are based on the recognition that the organisms that are used pose no health threat to humans or higher animals (unless deliberately modified for that purpose), and that the containment conditions minimize the possibility of an unanticipated deleterious effect on organisms and ecosystems outside of the experimental facility, e.g., the inadvertent spread of a serious pathogen from a greenhouse to a local agricultural crop or the unintentional introduction and establishment of an organism in a new ecosystem.

Appendix P-I-C. Four biosafety levels, referred to as Biosafety Level (BL) 1 - Plants (P), BL2-P, BL3-P, and BL4-P, are established in Appendix P-II, *Physical Containment Levels*. The selection of containment levels required for research involving recombinant or synthetic nucleic acid molecules in plants or associated with plants is specified in Appendix P-III, *Biological Containment Practices*. These biosafety levels are described in Appendix P-II, *Physical Containment Levels*. This appendix describes greenhouse practices and special greenhouse facilities for physical containment.

Appendix P-I-D. BL1-P through BL4-P are designed to provide differential levels of biosafety for plants in the absence or presence of other experimental organisms that contain recombinant or synthetic nucleic acid

molecules. These biosafety levels, in conjunction with biological containment conditions described in Appendix P-III, *Biological Containment Practices*, provide flexible approaches to ensure the safe conduct of research.

Appendix P-I-E. For experiments in which plants are grown at the BL1 through BL4 laboratory settings, containment practices shall be followed as described in Appendix G, *Physical Containment*. These containment practices include the use of plant tissue culture rooms, growth chambers within laboratory facilities, or experiments performed on open benches. Additional biological containment practices should be added by the Greenhouse Director or Institutional Biosafety Committee as necessary (see Appendix P-III, *Biological Containment Practices*), if botanical reproductive structures are produced that have the potential of being released.

Appendix P-II. Physical Containment Levels

Appendix P-II-A. Biosafety Level 1 - Plants (BL1-P)

Appendix P-II-A-1. Standard Practices (BL1-P)

Appendix P-II-A-1-a. Greenhouse Access (BL1-P)

Appendix P-II-A-1-a-(1). Access to the greenhouse shall be limited or restricted, at the discretion of the Greenhouse Director, when experiments are in progress.

Appendix P-II-A-1-a-(2). Prior to entering the greenhouse, personnel shall be required to read and follow instructions on BL1-P greenhouse practices and procedures. All procedures shall be performed in accordance with accepted greenhouse practices that are appropriate to the experimental organism.

Appendix P-II-A-1-b. Records (BL1-P)

Appendix P-II-A-1-b-(1). A record shall be kept of experiments currently in progress in the greenhouse facility.

Appendix P-II-A-1-c. Decontamination and Inactivation (BL1-P)

Appendix P-II-A-1-c-(1). Experimental organisms shall be rendered biologically inactive by appropriate methods before disposal outside of the greenhouse facility.

Appendix P-II-A-1-d. Control of Undesired Species and Motile Macroorganisms (BL1-P)

Appendix P-II-A-1-d-(1). A program shall be implemented to control undesired species (e.g., weed, rodent, or arthropod pests and pathogens), by methods appropriate to the organisms and in accordance with applicable state and Federal laws.

Appendix P-II-A-1-d-(2). Arthropods and other motile macroorganisms shall be housed in appropriate cages. If macroorganisms (e.g., flying arthropods or nematodes) are released within the greenhouse, precautions shall be taken to minimize escape from the greenhouse facility.

Appendix P-II-A-1-e. Concurrent Experiments Conducted in the Greenhouse (BL1-P)

Appendix P-II-A-1-e-(1). Experiments involving other organisms that require a containment level lower than BL1-P may be conducted in the greenhouse concurrently with experiments that require BL1-P containment, provided that all work is conducted in accordance with BL1-P greenhouse practices.

Appendix P-II-A-2. Facilities (BL1-P)

Appendix P-II-A-2-a. Definitions (BL1-P)

Appendix P-II-A-2-a-(1). The term "greenhouse" refers to a structure with walls, a roof, and a floor designed and used principally for growing plants in a controlled and protected environment. The walls and roof are usually constructed of transparent or translucent material to allow passage of sunlight for plant growth.

Appendix P-II-A-2-a-(2). The term "greenhouse facility" includes the actual greenhouse rooms or compartments for growing plants, including all immediately contiguous hallways and head-house areas, and is considered part of the confinement area.

Appendix P-II-A-2-b. Greenhouse Design (BL1-P)

Appendix P-II-A-2-b-(1). The greenhouse floor may be composed of gravel or other porous material. At a minimum, impervious (e.g., concrete) walkways are recommended.

Appendix P-II-A-2-b-(2). Windows and other openings in the walls and roof of the greenhouse facility may be open for ventilation as needed for proper operation and do not require any special barrier to contain or exclude pollen, microorganisms, or small flying animals (e.g., arthropods and birds); however, screens are recommended.

Appendix P-II-B. Biosafety Level 2 - Plants (BL2-P)

Appendix P-II-B-1. Standard Practices (BL2-P)

Appendix P-II-B-1-a. Greenhouse Access (BL2-P)

Appendix P-II-B-1-a-(1). Access to the greenhouse shall be limited or restricted, at the discretion of the Greenhouse Director, to individuals directly involved with the experiments when they are in progress.

Appendix P-II-B-1-a-(2). Personnel shall be required to read and follow instructions on BL2-P practices and procedures. All procedures shall be conducted in accordance with accepted greenhouse practices that are appropriate to the experimental organisms.

Appendix P-II-B-1-b. Records (BL2-P)

Appendix P-II-B-1-b-(1). A record shall be kept of experimental plants, microorganisms, or small animals that are brought into or removed from the greenhouse facility.

Appendix P-II-B-1-b-(2). A record shall be kept of experiments currently in progress in the greenhouse facility.

Appendix P-II-B-1-b-(3). The Principal Investigator shall report any greenhouse accident involving the inadvertent release or spill of microorganisms to the Greenhouse Director, Institutional Biosafety Committee, NIH/OBA and other appropriate authorities immediately (if applicable). Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Documentation of any such accident shall be prepared and maintained.

Appendix P-II-B-1-c. Decontamination and Inactivation (BL2-P)

Appendix P-II-B-1-c-(1). Experimental organisms shall be rendered biologically inactive by appropriate methods before disposal outside of the greenhouse facility.

Appendix P-II-B-1-c-(2). Decontamination of run-off water is not necessarily required. If part of the greenhouse is composed of gravel or similar material, appropriate treatments should be made periodically to eliminate, or render inactive, any organisms potentially entrapped by the gravel.

Appendix P-II-B-1-d. Control of Undesired Species and Motile Macroorganisms (BL2-P)

Appendix P-II-B-1-d-(1). A program shall be implemented to control undesired species (e.g., weed, rodent, or arthropod pests and pathogens) by methods appropriate to the organisms and in accordance with applicable state and Federal laws.

Appendix P-II-B-1-d-(2). Arthropods and other motile macroorganisms shall be housed in appropriate cages. If macroorganisms (e.g., flying arthropods or nematodes) are released within the greenhouse, precautions shall be taken to minimize escape from the greenhouse facility.

Appendix P-II-B-1-e. Concurrent Experiments Conducted in the Greenhouse (BL2-P)

Appendix P-II-B-1-e-(1). Experiments involving other organisms that require a containment level lower than BL2-P may be conducted in the greenhouse concurrently with experiments that require BL2-P containment provided that all work is conducted in accordance with BL2-P greenhouse practices.

Appendix P-II-B-1-f. Signs (BL2-P)

Appendix P-II-B-1-f-(1). A sign shall be posted indicating that a restricted experiment is in progress. The sign shall indicate the following: (i) the name of the responsible individual, (ii) the plants in use, and (iii) any special requirements for using the area.

Appendix P-II-B-1-f-(2). If organisms are used that have a recognized potential for causing serious detrimental impacts on managed or natural ecosystems, their presence shall be indicated on a sign posted on the greenhouse access doors.

Appendix P-II-B-1-f-(3). If there is a risk to human health, a sign shall be posted incorporating the universal biosafety symbol.

Appendix P-II-B-1-g. Transfer of Materials (BL2-P)

Appendix P-II-B-1-g-(1). Materials containing experimental microorganisms, which are brought into or removed from the greenhouse facility in a viable or intact state, shall be transferred in a closed non-breakable container.

Appendix P-II-B-1-h. Greenhouse Practices Manual (BL2-P)

Appendix P-II-B-1-h-(1). A greenhouse practices manual shall be prepared or adopted. This manual shall: (i) advise personnel of the potential consequences if such practices are not followed, and (ii) outline contingency plans to be implemented in the event of the unintentional release of organisms.

Appendix P-II-B-2. Facilities (BL2-P)

Appendix P-II-B-2-a. Definitions (BL2-P)

Appendix P-II-B-2-a-(1). The term "greenhouse" refers to a structure with walls, a roof, and a floor designed and used principally for growing plants in a controlled and protected environment. The walls and roof are usually constructed of transparent or translucent material to allow passage of sunlight for plant growth.

Appendix P-II-B-2-a-(2). The term "greenhouse facility" includes the actual greenhouse rooms or compartments for growing plants, including all immediately contiguous hallways and head-house areas and is considered part of the confinement area.

Appendix P-II-B-2-b. Greenhouse Design (BL2-P)

Appendix P-II-B-2-b-(1). A greenhouse floor composed of an impervious material. Concrete is recommended, but gravel or other porous material under benches is acceptable unless propagules of experimental organisms are readily disseminated through soil. Soil beds are acceptable unless propagules of experimental organisms are readily disseminated through soil.

Appendix P-II-B-2-b-(2). Windows and other openings in the walls and roof of the greenhouse facility may be open for ventilation as needed for proper operation and do not require any special barrier to exclude pollen or microorganisms; however, screens are required to exclude small flying animals (e.g., arthropods and birds).

Appendix P-II-B-2-c. Autoclaves (BL2-P)

Appendix P-II-B-2-c-(1). An autoclave shall be available for the treatment of contaminated greenhouse materials.

Appendix P-II-B-2-d. Supply and Exhaust Air Ventilation Systems (BL2-P)

Appendix P-II-B-2-d-(1). If intake fans are used, measures shall be taken to minimize the ingress of arthropods. Louvers or fans shall be constructed such that they can only be opened when the fan is in operation.

Appendix P-II-B-2-e. Other (BL2-P)

Appendix P-II-B-2-e-(1). BL2-P greenhouse containment requirements may be satisfied by using a growth chamber or growth room within a building provided that the external physical structure limits access and escape of microorganisms and macroorganisms in a manner that satisfies the intent of the foregoing clauses.

Appendix P-II-C. Biosafety Level 3 - Plants (BL3-P)

Appendix P-II-C-1. Standard Practices (BL3-P)

Appendix P-II-C-1-a. Greenhouse Access (BL3-P)

Appendix P-II-C-1-a-(1). Authorized entry into the greenhouse shall be restricted to individuals who are required for program or support purposes. The Greenhouse Director shall be responsible for assessing each circumstance and determining those individuals who are authorized to enter the greenhouse facility.

Appendix P-II-C-1-a-(2). Prior to entering the greenhouse, personnel shall be required to read and follow instructions on BL3-P practices and procedures. All procedures shall be conducted in accordance with accepted greenhouse practices that are appropriate to the experimental organisms.

Appendix P-II-C-1-b. Records (BL3-P)

Appendix P-II-C-1-b-(1). A record shall be kept of experimental plants, microorganisms, or small animals that are brought into or removed from the greenhouse facility.

Appendix P-II-C-1-b-(2). A record shall be kept of experiments currently in progress in the greenhouse facility.

Appendix P-II-C-1-b-(3). The Principal Investigator shall report any greenhouse accident involving the inadvertent release or spill of microorganisms to the Biological Safety Officer, Greenhouse Director, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities immediately (if applicable). Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Documentation of any such accident shall be prepared and maintained.

Appendix P-II-C-1-c. Decontamination and Inactivation (BL3-P)

Appendix P-II-C-1-c-(1). All experimental materials shall be sterilized in an autoclave or rendered biologically inactive by appropriate methods before disposal, except those that are to remain in a viable or intact state for experimental purposes; including water that comes in contact with experimental microorganisms or with material exposed to such microorganisms, and contaminated equipment and supplies.

Appendix P-II-C-1-d. Control of Undesired Species and Motile Macroorganisms (BL3-P)

Appendix P-II-C-1-d-(1). A program shall be implemented to control undesired species (e.g., weed, rodent, or arthropod pests and pathogens) by methods appropriate to the organisms and in accordance with applicable state and Federal laws.

Appendix P-II-C-1-d-(2). Arthropods and other motile macroorganisms shall be housed in appropriate cages. When appropriate to the organism, experiments shall be conducted within cages designed to contain the motile organisms.

Appendix P-II-C-1-e. Concurrent Experiments Conducted in the Greenhouse (BL3-P)

Appendix P-II-C-1-e-(1). Experiments involving organisms that require a containment level lower than BL3-P may be conducted in the greenhouse concurrently with experiments that require BL3-P containment provided that all work is conducted in accordance with BL3-P greenhouse practices.

Appendix P-II-C-1-f. Signs (BL3-P)

Appendix P-II-C-1-f-(1). A sign shall be posted indicating that a restricted experiment is in progress. The sign shall indicate the following: (i) the name of the responsible individual, (ii) the plants in use, and (iii) any special requirements for using the area.

Appendix P-II-C-1-f-(2). If organisms are used that have a recognized potential for causing serious detrimental impacts on managed or natural ecosystems, their presence should be indicated on a sign posted on the greenhouse access doors.

Appendix P-II-C-1-f-(3). If there is a risk to human health, a sign shall be posted incorporating the universal biosafety symbol.

Appendix P-II-C-1-g. Transfer of Materials (BL3-P)

Appendix P-II-C-1-g-(1). Experimental materials that are brought into or removed from the greenhouse facility in a viable or intact state shall be transferred to a non-breakable sealed secondary container. At the time of transfer, if the same plant species, host, or vector are present within the effective dissemination distance of propagules of the experimental organism, the surface of the secondary container shall be decontaminated. Decontamination may be accomplished by passage through a chemical disinfectant or fumigation chamber or by an alternative procedure that has demonstrated effective inactivation of the experimental organism.

Appendix P-II-C-1-h. Greenhouse Practices Manual (BL3-P)

Appendix P-II-C-1-h-(1). A greenhouse practices manual shall be prepared or adopted. This manual shall: (i) advise personnel of the potential consequences if such practices are not followed, and (ii) outline contingency plans to be implemented in the event of the unintentional release of organisms with recognized potential for serious detrimental impact.

Appendix P-II-C-1-i. Protective Clothing (BL3-P)

Appendix P-II-C-1-i-(1). Disposable clothing (e.g., solid front or wrap-around gowns, scrub suits, or other appropriate clothing) shall be worn in the greenhouse if deemed necessary by the Greenhouse Director because of potential dissemination of the experimental microorganisms.

Appendix P-II-C-1-i-(2). Protective clothing shall be removed before exiting the greenhouse and decontaminated prior to laundering or disposal.

Appendix P-II-C-1-j. Other (BL3-P)

Appendix P-II-C-1-j-(1). Personnel are required to thoroughly wash their hands upon exiting the greenhouse.

Appendix P-II-C-1-j-(2). All procedures shall be performed carefully to minimize the creation of aerosols and excessive splashing of potting material/soil during watering, transplanting, and all experimental manipulations.

Appendix P-II-C-2. Facilities (BL3-P)

Appendix P-II-C-2-a. Definitions (BL3-P)

Appendix P-II-C-2-a-(1). The term "greenhouse" refers to a structure with walls, roof, and floor designed and used principally for growing plants in a controlled and protected environment. The walls and roof are usually constructed of transparent or translucent material to allow passage of sunlight for plant growth.

Appendix P-II-C-2-a-(2). The term "greenhouse facility" includes the actual greenhouse rooms or compartments for growing plants, including all immediately contiguous hallways and head-house areas, and is

considered part of the confinement area. The need to maintain negative pressure should be considered when constructing or renovating the greenhouse.

Appendix P-II-C-2-b. Greenhouse Design (BL3-P)

Appendix P-II-C-2-b-(1). The greenhouse floor shall be composed of concrete or other impervious material with provision for collection and decontamination of liquid run-off.

Appendix P-II-C-2-b-(2). Windows shall be closed and sealed. All glazing shall be resistant to breakage (e.g., double-pane tempered glass or equivalent).

Appendix P-II-C-2-b-(3). The greenhouse shall be a closed self-contained structure with a continuous covering that is separated from areas that are open to unrestricted traffic flow. The minimum requirement for greenhouse entry shall be passage through two sets of self-closing locking doors.

Appendix P-II-C-2-b-(4). The greenhouse facility shall be surrounded by a security fence or protected by equivalent security measures.

Appendix P-II-C-2-b-(5). Internal walls, ceilings, and floors shall be resistant to penetration by liquids and chemicals to facilitate cleaning and decontamination of the area. All penetrations into these structures and surfaces (e.g., plumbing and utilities) shall be sealed.

Appendix P-II-C-2-b-(6). Bench tops and other work surfaces should have seamless surfaces that are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix P-II-C-2-b-(7). The greenhouse contains a foot, elbow, or automatically operated sink, which is located near the exit door for hand washing.

Appendix P-II-C-2-c. Autoclaves (BL3-P)

Appendix P-II-C-2-c-(1). An autoclave shall be available for decontaminating materials within the greenhouse facility. A double-door autoclave is recommended (not required) for the decontamination of materials passing out of the greenhouse facility.

Appendix P-II-C-2-d. Supply and Exhaust Air Ventilation Systems (BL3-P)

Appendix P-II-C-2-d-(1). An individual supply and exhaust air ventilation system shall be provided. The system maintains pressure differentials and directional airflow, as required, to assure inward (or zero) airflow from areas outside of the greenhouse.

Appendix P-II-C-2-d-(2). The exhaust air from the greenhouse facility shall be filtered through high efficiency particulate air-HEPA filters and discharged to the outside. The filter chambers shall be designed to allow *in situ* decontamination before filters are removed and to facilitate certification testing after they are replaced. Air filters shall be 80-85% average efficiency by the American Society of Heating, Refrigerating, and Air Conditioning Engineers (ASHRAE) Standard 52-68 test method using atmosphere dust. Air supply fans shall be equipped with a back-flow damper that closes when the air supply fan is off. Alternatively, a HEPA filter may be used on the air supply system instead of the filters and damper. The supply and exhaust airflow shall be interlocked to assure inward (or zero) airflow at all times.

Appendix P-II-C-2-e. Other (BL3-P)

Appendix P-II-C-2-e-(1). BL3-P greenhouse containment requirements may be satisfied using a growth chamber or growth room within a building provided that the location, access, airflow patterns, and provisions for decontamination of experimental materials and supplies meet the intent of the foregoing clauses.

Appendix P-II-C-2-e-(2). Vacuum lines shall be protected with high efficiency particulate air/HEPA or equivalent filters and liquid disinfectant traps.

Appendix P-II-D. Biosafety Level 4 - Plants (BL4-P)

Appendix P-II-D-1. Standard Practices (BL4-P)

Appendix P-II-D-1-a. Greenhouse Access (BL4-P)

Appendix P-II-D-1-a-(1). Authorized entry into the greenhouse shall be restricted to individuals who are required for program or support purposes. The Greenhouse Director shall be responsible for assessing each circumstance and determining those individuals who are authorized to enter the greenhouse facility or work in the greenhouse during experiments.

Appendix P-II-D-1-a-(2). Access shall be managed by the Greenhouse Director, Biological Safety Officer, or other individual responsible for physical security of the greenhouse facility; and access limited by means of secure, locked doors.

Appendix P-II-D-1-a-(3). Prior to entering, individuals shall be advised of the potential environmental hazards and instructed on appropriate safeguards for ensuring environmental safety. Individuals authorized to enter the greenhouse facility shall comply with the instructions and all other applicable entry/exit procedures.

Appendix P-II-D-1-a-(4). Personnel shall enter and exit the greenhouse facility only through the clothing change and shower rooms and shall shower each time they exit the greenhouse facility. Personnel shall use the airlocks to enter or exit the laboratory only in an emergency. In the event of an emergency, every reasonable effort should be made to prevent the possible transport of viable propagules from containment.

Appendix P-II-D-1-a-(5). Prior to entering the greenhouse, personnel shall be required to read and follow instructions on BL4-P practices and procedures.

Appendix P-II-D-1-b. Records (BL4-P)

Appendix P-II-D-1-b-(1). A record shall be kept of all experimental materials brought into or removed from the greenhouse.

Appendix P-II-D-1-b-(2). A record shall be kept of experiments currently in progress in the greenhouse facility.

Appendix P-II-D-1-b-(3). A record shall be kept of all personnel entering and exiting the greenhouse facility, including the date and time of each entry.

Appendix P-II-D-1-b-(4). The Principal Investigator shall report any greenhouse accident involving the inadvertent release or spill of microorganisms to the Biological Safety Officer, Greenhouse Director, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities immediately (if applicable). Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Documentation of any such accident shall be prepared and maintained.

Appendix P-II-D-1-c. Decontamination and Inactivation (BL4-P)

Appendix P-II-D-1-c-(1). All materials, except for those that are to remain in a viable or intact state for experimental purposes, shall be autoclaved prior to removal from the maximum containment greenhouse. Equipment or material that could be damaged by high temperatures or steam shall be decontaminated by alternative methods (e.g., gas or vapor sterilization) in an airlock or chamber designed for this purpose.

Appendix P-II-D-1-c-(2). Water that comes in contact with experimental microorganisms or with material exposed to such microorganisms (e.g., run-off from watering plants) shall be collected and decontaminated before disposal.

Appendix P-II-D-1-c-(3). Standard microbiological procedures shall be followed for decontamination of equipment and materials. Spray or liquid waste or rinse water from containers used to apply the experimental microorganisms shall be decontaminated before disposal.

Appendix P-II-D-1-d. Control of Undesired Species and Motile Macroorganisms (BL4-P)

Appendix P-II-D-1-d-(1). A chemical control program shall be implemented to eliminate undesired pests and pathogens in accordance with applicable state and Federal laws.

Appendix P-II-D-1-d-(2). Arthropods and other motile macroorganisms used in conjunction with experiments requiring BL4-P level physical containment shall be housed in appropriate cages. When appropriate to the organism, experiments shall be conducted within cages designed to contain the motile organisms.

Appendix P-II-D-1-e. Concurrent Experiments Conducted in the Greenhouse (BL4-P)

Appendix P-II-D-1-e-(1). Experiments involving organisms that require a containment level lower than BL4-P may be conducted in the greenhouse concurrently with experiments that require BL4-P containment provided that all work is conducted in accordance with BL4-P greenhouse practices. When the experimental microorganisms in use require a containment level lower than BL4-P, greenhouse practices reflect the level of containment required by the highest containment level microorganisms being tested.

Appendix P-II-D-1-f. Signs (BL4-P)

Appendix P-II-D-1-f-(1). A sign shall be posted indicating that a restricted experiment is in progress. The sign shall indicate the following: (i) the name of the responsible individual, (ii) the plants in use, and (iii) any special requirements for using the area.

Appendix P-II-D-1-f-(2). If organisms are used that have a recognized potential for causing serious detrimental impacts on managed or natural ecosystems, their presence shall be indicated by a sign posted on the greenhouse access doors.

Appendix P-II-D-1-f-(3). If there is a risk to human health, a sign shall be posted incorporating the universal biosafety symbol.

Appendix P-II-D-1-g. Transfer of Materials (BL4-P)

Appendix P-II-D-1-g-(1). Experimental materials that are brought into or removed from the greenhouse in a viable or intact state shall be transferred to a non-breakable, sealed, primary container then enclosed in a non-breakable, sealed secondary container. These containers shall be removed from the greenhouse facility through a chemical disinfectant, fumigation chamber, or an airlock designed for this purpose.

Appendix P-II-D-1-g-(2). Supplies and materials shall be brought into the greenhouse facility through a double-door autoclave, fumigation chamber, or airlock that is appropriately decontaminated between each use. After securing the outer doors, personnel within the greenhouse facility shall retrieve the materials by opening the interior door of the autoclave, fumigation chamber, or airlock. These doors shall be secured after the materials are brought into the greenhouse facility.

Appendix P-II-D-1-h. Greenhouse Practices Manual (BL4-P)

Appendix P-II-D-1-h-(1). A greenhouse practices manual shall be prepared or adopted. This manual shall include contingency plans to be implemented in the event of the unintentional release of experimental organisms.

Appendix P-II-D-1-i. Protective Clothing (BL4-P)

Appendix P-II-D-1-i-(1). Street clothing shall be removed in the outer clothing change room. Complete laboratory clothing (may be disposable) including undergarments, pants, and shirts, jump suits, shoes, and hats shall be provided and worn by all personnel entering the greenhouse facility.

Appendix P-II-D-1-i-(2). Personnel shall remove laboratory clothing when exiting the greenhouse facility and before entering the shower area. This clothing shall be stored in a locker or hamper in the inner change room.

Appendix P-II-D-1-i-(3). All laboratory clothing shall be autoclaved before laundering.

Appendix P-II-D-2. Facilities (BL4-P)

Appendix P-II-D-2-a. Greenhouse Design (BL4-P)

Appendix P-II-D-2-a-(1). The maximum containment greenhouse facility shall consist of a separate building or a clearly demarcated and isolated area within a building. The need to maintain negative pressure should be considered when constructing or renovating the greenhouse facility.

Appendix P-II-D-2-a-(2). Outer and inner change rooms, separated by a shower, shall be provided for personnel entering and exiting the greenhouse facility.

Appendix P-II-D-2-a-(3). Windows shall be closed and sealed. All glazing shall be resistant to breakage (e.g., double-pane tempered glass or equivalent).

Appendix P-II-D-2-a-(4). Access doors to the greenhouse shall be self-closing and locking.

Appendix P-II-D-2-a-(5). The greenhouse facility shall be surrounded by a security fence or protected by equivalent security measures.

Appendix P-II-D-2-a-(6). The walls, floors, and ceilings of the greenhouse shall be constructed to form a sealed internal shell that facilitates fumigation and is animal and arthropod-proof. These internal surfaces shall be resistant to penetration and degradation by liquids and chemicals to facilitate cleaning and decontamination of the area. All penetrations into these structures and surfaces (e.g., plumbing and utilities) shall be sealed.

Appendix P-II-D-2-a-(7). Bench tops and other work surfaces shall have seamless surfaces impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix P-II-D-2-a-(8). A double-door autoclave, fumigation chamber, or ventilated airlock shall be provided for passage of all materials, supplies, or equipment that are not brought into the greenhouse facility through the change room.

Appendix P-II-D-2-b. Autoclaves (BL4-P)

Appendix P-II-D-2-b-(1). A double-door autoclave shall be provided for the decontamination of materials removed from the greenhouse facility. The autoclave door, which opens to the area external to the greenhouse facility, shall be sealed to the outer wall and automatically controlled so that it can only be opened upon completion of the sterilization cycle.

Appendix P-II-D-2-c. Supply and Exhaust Air Ventilation Systems (BL4-P)

Appendix P-II-D-2-c-(1). An individual supply and exhaust air ventilation system shall be provided. The system shall maintain pressure differentials and directional airflow as required to assure inward (or zero) airflow from areas outside of the greenhouse. Differential pressure transducers shall be used to sense pressure levels. If a system malfunctions, the transducers shall sound an alarm. A backup source of power should be considered. The supply and exhaust airflow shall be interlocked to assure inward (or zero) airflow at all times. The integrity of the greenhouse shall have an air leak rate (decay rate) not to exceed 7 percent per minute (logarithm of pressure against time) over a 20-minute period at 2 inches of water gauge pressure. Nominally, this is 0.05 inches of water gauge pressure loss in 1 minute at 2 inches water gauge pressure.

Appendix P-II-D-2-c-(2). Exhaust air from the greenhouse facility shall be filtered through high efficiency particulate air/HEPA filters and discharged to the outside and dispersed away from occupied buildings and air intakes. Filter chambers shall be designed to allow *in situ* decontamination before filters are removed and to facilitate certification testing after they are replaced. HEPA filters shall be provided to treat air supplied to the greenhouse facility. HEPA filters shall be certified annually.

Appendix P-II-D-2-d. Other (BL4-P)

Appendix P-II-D-2-d-(1). Sewer vents and other ventilation lines contain high efficiency particulate air/HEPA filters. HEPA filters shall be certified annually.

Appendix P-II-D-2-d-(2). A pass-through dunk tank, fumigation chamber, or an equivalent method of decontamination shall be provided to ensure decontamination of materials and equipment that cannot be decontaminated in the autoclave.

Appendix P-II-D-2-d-(3). Liquid effluent from sinks, floors, and autoclave chambers shall be decontaminated by heat or chemical treatment before being released from the maximum containment greenhouse facility. Liquid wastes from shower rooms and toilets may be decontaminated by heat or chemical treatment. Autoclave and chemical decontamination of liquid wastes shall be evaluated by appropriate standard procedures for autoclaved wastes. Decontamination shall be evaluated mechanically and biologically using a recording thermometer and an indicator microorganism with a defined heat susceptibility pattern. If liquid wastes are decontaminated with chemical disinfectants, the chemicals used must have demonstrated efficacy against the target or indicator microorganisms.

Appendix P-II-D-2-d-(4). If there is a central vacuum system, it shall not serve areas outside of the greenhouse facility. In-line high efficiency particulate air/HEPA filters shall be placed as near as practicable to each use point or vacuum service cock. Other liquid and gas services to the greenhouse facility shall be protected by devices that prevent back-flow. HEPA filters shall be certified annually.

Appendix P-III. Biological Containment Practices

Appropriate selection of the following biological containment practices may be used to meet the containment requirements for a given organism. The present list is not exhaustive; there may be other ways of preventing effective dissemination that could possibly lead to the establishment of the organism or its genetic material in the environment resulting in deleterious consequences to managed or natural ecosystems.

Appendix P-III-A. Biological Containment Practices (Plants)

Appendix P-III-A-1. Effective dissemination of plants by pollen or seed can be prevented by one or more of the following procedures: (i) cover the reproductive structures to prevent pollen dissemination at flowering and seed dissemination at maturity; (ii) remove reproductive structures by employing male sterile strains, or harvest the plant material prior to the reproductive stage; (iii) ensure that experimental plants flower at a time of year when cross-fertile plants are not flowering within the normal pollen dispersal range of the experimental plant; or (iv) ensure that cross-fertile plants are not growing within the known pollen dispersal range of the experimental plant.

Appendix P-III-B. Biological Containment Practices (Microorganisms)

Appendix P-III-B-1. Effective dissemination of microorganisms beyond the confines of the greenhouse can be prevented by one or more of the following procedures: (i) confine all operations to injections of microorganisms or other biological procedures (including genetic manipulation) that limit replication or reproduction of viruses and microorganisms or sequences derived from microorganisms, and confine these injections to internal plant parts or adherent plant surfaces; (ii) ensure that organisms, which can serve as hosts or promote the transmission of the virus or microorganism, are not present within the farthest distance that the airborne virus or microorganism may be expected to be effectively disseminated; (iii) conduct experiments at a time of year when plants that can serve as hosts are either not growing or are not susceptible to productive infection; (iv) use viruses and other microorganisms or their genomes that have known arthropod or animal vectors, in the absence of such vectors; (v) use microorganisms that have an obligate association with the plant; or (vi) use microorganisms that are genetically disabled to minimize survival outside of the research facility and whose natural mode of transmission requires injury of the target organism, or assures that inadvertent release is unlikely to initiate productive infection of organisms outside of the experimental facility.

Appendix P-III-C. Biological Containment Practices (Macroorganisms)

Appendix P-III-C-1. Effective dissemination of arthropods and other small animals can be prevented by using one or more of the following procedures: (i) use non-flying, flight-impaired, or sterile arthropods; (ii) use non-motile or sterile strains of small animals; (iii) conduct experiments at a time of year that precludes the survival of escaping organisms; (iv) use animals that have an obligate association with a plant that is not present within the dispersal range of the organism; or (v) prevent the escape of organisms present in run-off water by chemical treatment or evaporation of run-off water.

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APPENDIX Q. PHYSICAL AND BIOLOGICAL CONTAINMENT FOR RECOMBINANT OR SYNTHETIC NUCLEIC ACID MOLECULE RESEARCH INVOLVING ANIMALS

Appendix Q specifies containment and confinement practices for research involving whole animals, both those in which the animal's genome has been altered by stable introduction of recombinant or synthetic nucleic acid molecules, or DNA derived therefrom, into the germ-line (transgenic animals) and experiments involving viable recombinant or synthetic nucleic acid molecule-modified microorganisms tested on whole animals. The appendix applies to animal research activities with the following modifications:

Appendix Q shall supersede Appendix G (*Physical Containment*) when research animals are of a size or have growth requirements that preclude the use of containment for laboratory animals. Some animals may require other types of containment (see Appendix Q-III-D, *Footnotes and References for Appendix Q*). The animals covered in Appendix Q are those species normally categorized as animals including but not limited to cattle, swine, sheep, goats, horses, and poultry.

The Institutional Biosafety Committee shall include at least one scientist with expertise in animal containment principles when experiments utilizing Appendix Q require Institutional Biosafety Committee prior approval.

The institution shall establish and maintain a health surveillance program for personnel engaged in animal research involving viable recombinant or synthetic nucleic acid molecule-containing microorganisms that require Biosafety Level (BL) 3 or greater containment in the laboratory.

Appendix Q-I. General Considerations

Appendix Q-I-A. Containment Levels

The containment levels required for research involving recombinant or synthetic nucleic acid molecules associated with or in animals is based on classification of experiments in Section III, *Experiments Covered by the NIH Guidelines*. For the purpose of animal research, four levels of containment are established. These are referred to as BL1-Animals (N), BL2-N, BL3-N, and BL4-N and are described in the following appendices of Appendix Q. The descriptions include: (i) standard practices for physical and biological containment, and (ii) animal facilities.

Appendix Q-I-B. Disposal of Animals (BL1-N through BL4-N)

Appendix Q-I-B-1. When an animal covered by Appendix Q containing recombinant or synthetic nucleic acid molecules or a recombinant or synthetic nucleic acid molecule-derived organism is euthanized or dies, the carcass shall be disposed of to avoid its use as food for human beings or animals unless food use is specifically authorized by an appropriate Federal agency.

Appendix Q-I-B-2. A permanent record shall be maintained of the experimental use and disposal of each animal or group of animals.

Appendix Q-II. Physical and Biological Containment Levels

Appendix Q-II-A. Biosafety Level 1 - Animals (BL1-N)

Appendix Q-II-A-1. Standard Practices (BL1-N)

Appendix Q-II-A-1-a. Animal Facility Access (BL1-N)

Appendix Q-II-A-1-a-(1). The containment area shall be locked.

Appendix Q-II-A-1-a-(2). Access to the containment area shall be limited or restricted when experimental animals are being held.

Appendix Q-II-A-1-a-(3). The containment area shall be patrolled or monitored at frequent intervals.

Appendix Q-II-A-1-b. Other (BL1-N)

Appendix Q-II-A-1-b-(1). All genetically engineered neonates shall be permanently marked within 72 hours after birth, if their size permits. If their size does not permit marking, their containers should be marked. In addition, transgenic animals should contain distinct and biochemically assayable DNA sequences that allow identification of transgenic animals from among non-transgenic animals.

Appendix Q-II-A-1-b-(2) A double barrier shall be provided to separate male and female animals unless reproductive studies are part of the experiment or other measures are taken to avoid reproductive transmission. Reproductive incapacitation may be used.

Appendix Q-II-A-1-b-(3). The containment area shall be in accordance with state and Federal laws and animal care requirements.

Appendix Q-II-A-2. Animal Facilities (BL1-N)

Appendix Q-II-A-2-a. Animals shall be confined to securely fenced areas or be in enclosed structures (animal rooms) to minimize the possibility of theft or unintentional release.

Appendix Q-II-B. Biosafety Level 2 - Animals (BL2-N) (See Appendix Q-III-A, *Footnotes and References for Appendix Q*)

Appendix Q-II-B-1. Standard Practices (BL2-N)

Appendix Q-II-B-1-a. Animal Facility Access (BL2-N)

Appendix Q-II-B-1-a-(1). The containment area shall be locked.

Appendix Q-II-B-1-a-(2). The containment area shall be patrolled or monitored at frequent intervals.

Appendix Q-II-B-1-a-(3). The containment building shall be controlled and have a locking access.

Appendix Q-II-B-1-a-(4). The Animal Facility Director shall establish policies and procedures whereby only persons who have been advised of the potential hazard and who meet any specific entry requirements (e.g., vaccination) may enter the laboratory or animal rooms.

Appendix Q-II-B-1-a-(5). Animals of the same or different species, which are not involved in the work being performed, shall not be permitted in the animal area.

Appendix Q-II-B-1-b. Decontamination and Inactivation (BL2-N)

Appendix Q-II-B-1-b-(1). Contaminated materials that are decontaminated at a site away from the laboratory shall be placed in a closed durable leak-proof container prior to removal from the laboratory.

Appendix Q-II-B-1-b-(2). Needles and syringes shall be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix Q-II-B-1-c. Signs (BL2-N)

Appendix Q-II-B-1-c-(1). When the animal research requires special provisions for entry (e.g., vaccination), a warning sign incorporating the universal biosafety symbol shall be posted on all access doors to the animal work area. The sign shall indicate: (i) the agent, (ii) the animal species, (iii) the name and telephone number of the Animal Facility Director or other responsible individual, and (iv) any special requirements for entering the laboratory.

Appendix Q-II-B-1-d. Protective Clothing (BL2-N)

Appendix Q-II-B-1-d-(1). Laboratory coats, gowns, smocks, or uniforms shall be worn while in the animal area or attached laboratory. Before entering non-laboratory areas (e.g., cafeteria, library, administrative offices),

protective clothing shall be removed and kept in the work entrance area.

Appendix Q-II-B-1-d-(2). Special care shall be taken to avoid skin contamination with microorganisms containing recombinant or synthetic nucleic acid molecules. Impervious and/or protective gloves shall be worn when handling experimental animals and when skin contact with an infectious agent is unavoidable.

Appendix Q-II-B-1-e. Records (BL2-N)

Appendix Q-II-B-1-e-(1). Any incident involving spills and accidents that result in environmental release or exposures of animals or laboratory workers to organisms containing recombinant or synthetic nucleic acid molecules shall be reported immediately to the Animal Facility Director, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment shall be provided as appropriate and written records maintained. If necessary, the area shall be appropriately decontaminated.

Appendix Q-II-B-1-e-(2). When appropriate and giving consideration to the agent handled, baseline serum samples shall be collected and stored for animal care and other at-risk personnel. Additional serum specimens may be collected periodically depending on the agent handled and the function of the animal facility.

Appendix Q-II-B-1-f. Transfer of Materials (BL2-N)

Appendix Q-II-B-1-f-(1). Biological materials removed from the animal containment area in a viable or intact state shall be transferred to a non-breakable sealed primary container and then enclosed in a non-breakable sealed secondary container. All containers, primary and secondary, shall be disinfected before removal from the animal facility. Advance approval for transfer of material shall be obtained from the Animal Facility Director. Packages containing viable agents may only be opened in a facility having an equivalent or higher level of physical containment unless the agent is biologically inactivated or incapable of reproduction.

Appendix Q-II-B-1-g. Other (BL2-N)

Appendix Q-II-B-1-g-(1). All genetically engineered neonates shall be permanently marked within 72 hours after birth, if their size permits. If their size does not permit marking, their containers should be marked. In addition, transgenic animals should contain distinct and biochemically assayable DNA sequences that allow identification of transgenic animals from among non-transgenic animals.

Appendix Q-II-B-1-g-(2). Needles and syringes shall be used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) shall be used for the injection or aspiration of fluids containing organisms that contain recombinant or synthetic nucleic acid molecules. Extreme caution shall be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Following use, needles shall not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe. Needles and syringes shall be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix Q-II-B-1-g-(3). Appropriate steps should be taken to prevent horizontal transmission or exposure of laboratory personnel. If the agent used as a vector is known to be transmitted by a particular route (e.g., arthropods), special attention should be given to preventing spread by that route. In the absence of specific knowledge of a particular route of transmission, all potential means of horizontal transmission (e.g., arthropods, contaminated bedding, or animal waste, etc.) should be prevented.

Appendix Q-II-B-1-g-(4). Eating, drinking, smoking, and applying cosmetics shall not be permitted in the work area.

Appendix Q-II-B-1-g-(5). Individuals who handle materials and animals containing recombinant or synthetic nucleic acid molecules shall be required to wash their hands before exiting the containment area.

Appendix Q-II-B-1-g-(6). A double barrier shall be provided to separate male and female animals unless reproductive studies are part of the experiment or other measures are taken to avoid reproductive transmission.

Reproductive incapacitation may be used.

Appendix Q-II-B-1-g-(7). The containment area shall be in accordance with state and Federal laws and animal care requirements.

Appendix Q-II-B-1-g-(8). A biosafety manual shall be prepared or adopted. Personnel shall be advised of special hazards and required to read and follow instructions on practices and procedures.

Appendix Q-II-B-2. Animal Facilities (BL2-N)

Appendix Q-II-B-2-a. Animals shall be contained within an enclosed structure (animal room or equivalent) to minimize the possibility of theft or unintentional release and to avoid arthropod access. The special provision to avoid the entry or escape of arthropods from the animal areas may be waived if the agent in use is not known to be transmitted by arthropods.

Appendix Q-II-B-2-b. Surfaces shall be impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix Q-II-B-2-c. The animal containment area shall be designed so that it can be easily cleaned.

Appendix Q-II-B-2-d. Windows that open shall be fitted with fly screens.

Appendix Q-II-B-2-e. An autoclave shall be available for decontamination of laboratory wastes.

Appendix Q-II-B-2-f. If arthropods are used in the experiment or the agent under study can be transmitted by an arthropod, interior work areas shall be appropriately screened (52 mesh). All perimeter joints and openings shall be sealed and additional arthropod control mechanisms used to minimize arthropod entry and propagation, including appropriate screening of access doors or the equivalent.

Appendix Q-II-C. Biosafety Level 3 - Animals (BL3-N) (See Appendix Q-III-B, *Footnotes and References for Appendix Q*)

Appendix Q-II-C-1. Standard Practices (BL3-N)

Appendix Q-II-C-1-a. Animal Facility Access (BL3-N)

Appendix Q-II-C-1-a-(1). The containment area shall be locked.

Appendix Q-II-C-1-a-(2). The containment area shall be patrolled or monitored at frequent intervals.

Appendix Q-II-C-1-a-(3). The containment building shall be controlled and have a locking access.

Appendix Q-II-C-1-a-(4). The Animal Facility Director shall establish policies and procedures whereby only persons who have been advised of the potential hazard and who meet any specific entry requirements (e.g., vaccination) shall enter the laboratory or animal rooms.

Appendix Q-II-C-1-a-(5). Animal room doors, gates, or other closures shall be kept closed when experiments are in progress.

Appendix Q-II-C-1-b. Decontamination and Inactivation (BL3-N)

Appendix Q-II-C-1-b-(1). The work surfaces of containment equipment shall be decontaminated when work with organisms containing recombinant or synthetic nucleic acid molecules is finished. Where feasible, plastic-backed paper toweling shall be used on nonporous work surfaces to facilitate clean-up.

Appendix Q-II-C-1-b-(2). All animals shall be euthanized at the end of their experimental usefulness and the carcasses decontaminated before disposal in an approved manner.

Appendix Q-II-C-1-b-(3). Needles and syringes shall be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix Q-II-C-1-b-(4). Special safety testing, decontamination procedures, and Institutional Biosafety Committee approval shall be required to transfer agents or tissue/organ specimens from a BL3-N animal facility to a facility with a lower containment classification.

Appendix Q-II-C-1-b-(5). Liquid effluent from containment equipment, sinks, biological safety cabinets, animal rooms, primary barriers, floor drains, and sterilizers shall be decontaminated by heat treatment before being released into the sanitary system. The procedure used for heat decontamination of liquid wastes shall be monitored with a recording thermometer. The effectiveness of the heat decontamination process system shall be revalidated every 30 days with an indicator organism.

Appendix Q-II-C-1-c. Signs (BL3-N)

Appendix Q-II-C-1-c-(1). When the animal research requires special provisions for entry (e.g., vaccination), a warning sign incorporating the universal biosafety symbol shall be posted on all access doors to the animal work area. The sign shall indicate: (i) the agent, (ii) the animal species, (iii) the name and telephone number of the Animal Facility Director or other responsible individual, and (iv) any special requirements for entering the laboratory.

Appendix Q-II-C-1-d. Protective Clothing (BL3-N)

Appendix Q-II-C-1-d-(1). Full protective clothing that protects the individual (e.g., scrub suits, coveralls, uniforms) shall be worn in the animal area. Clothing shall not be worn outside the animal containment area and shall be decontaminated before laundering or disposal. Personnel shall be required to shower before exiting the BL3-N area and wearing of personal clothing.

Appendix Q-II-C-1-d-(2). Special care shall be taken to avoid skin contamination with microorganisms containing recombinant or synthetic nucleic acid molecules. Impervious and/or protective gloves shall be worn when handling experimental animals and when skin contact with an infectious agent is unavoidable.

Appendix Q-II-C-1-d-(3). Appropriate respiratory protection shall be worn in rooms containing experimental animals.

Appendix Q-II-C-1-e. Records (BL3-N)

Appendix Q-II-C-1-e-(1). Documents regarding experimental animal use and disposal shall be maintained in a permanent record book.

Appendix Q-II-C-1-e-(2). Any incident involving spills and accidents that result in environmental release or exposure of animals or laboratory workers to organisms containing recombinant or synthetic nucleic acid molecules shall be reported immediately to the Biological Safety Office, Animal Facility Director, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment shall be provided as appropriate and written records maintained. If necessary, the area shall be appropriately decontaminated.

Appendix Q-II-C-1-e-(3). When appropriate and giving consideration to the agent handled, baseline serum samples shall be collected and stored for animal care and other at-risk personnel. Additional serum specimens may be collected periodically depending on the agent handled or the function of the facility.

Appendix Q-II-C-1-f. Transfer of Materials (BL3-N)

Appendix Q-II-C-1-f-(1). Biological materials removed from the animal containment laboratory in a viable or intact state shall be transferred to a non-breakable sealed primary container and then enclosed in a non-breakable sealed secondary container. All containers, primary and secondary, shall be disinfected before removal from the animal facility. Advance approval for transfer of material shall be obtained from the Animal Facility Director. Packages containing viable agents may be opened only in a facility having an equivalent or higher level of physical containment unless the agent is biologically inactivated or incapable of reproduction.

Appendix Q-II-C-1-f-(2). Special safety testing, decontamination procedures, and Institutional Biosafety Committee approval shall be required to transfer agents or tissue/organ specimens from a BL3-N animal facility to a facility with a lower containment classification.

Appendix Q-II-C-1-g. Other (BL3-N)

Appendix Q-II-C-1-g-(1). All genetically engineered neonates shall be permanently marked within 72 hours after birth, if their size permits. If their size does not permit marking, their containers should be marked. In addition, transgenic animals should contain distinct and biochemically assayable DNA sequences that allow identification of transgenic animals from among non-transgenic animals.

Appendix Q-II-C-1-g-(2). Appropriate steps should be taken to prevent horizontal transmission or exposure of laboratory personnel. If the agent used as the vector is known to be transmitted by a particular route (e.g., arthropods), special attention should be given to preventing spread by that route. In the absence of specific knowledge of a particular route of transmission, all potential means of horizontal transmission (e.g., arthropods, contaminated bedding, or animal waste) should be prevented.

Appendix Q-II-C-1-g-(3). Eating, drinking, smoking, and applying cosmetics shall not be permitted in the work area.

Appendix Q-II-C-1-g-(4). Individuals who handle materials and animals containing recombinant or synthetic nucleic acid molecules shall be required to wash their hands before exiting the containment area.

Appendix Q-II-C-1-g-(5). Experiments involving other organisms that require containment levels lower than BL3-N may be conducted in the same area concurrently with experiments requiring BL3-N containment provided that they are conducted in accordance with BL3-N practices.

Appendix Q-II-C-1-g-(6). Animal holding areas shall be cleaned at least once a day and decontaminated immediately following any spill of viable materials.

Appendix Q-II-C-1-g-(7). All procedures shall be performed carefully to minimize the creation of aerosols.

Appendix Q-II-C-1-g-(8). A double barrier shall be provided to separate male and female animals unless reproductive studies are part of the experiment or other measures are taken to avoid reproductive transmission. Reproductive incapacitation may be used.

Appendix Q-II-C-1-g-(9). The containment area shall be in accordance with state and Federal laws and animal care requirements.

Appendix Q-II-C-1-g-(10). All animals shall be euthanized at the end of their experimental usefulness and the carcasses decontaminated before disposal in an approved manner.

Appendix Q-II-C-1-g-(11). Personnel shall be required to shower before exiting the BL3-N area and wearing personal clothing.

Appendix Q-II-C-1-g-(12). Animals of the same or different species, which are not involved in the work being performed, shall not be permitted in the animal area.

Appendix Q-II-C-1-g-(13). Needles and syringes shall be used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) shall be used for the injection or aspiration of fluids containing organisms that contain recombinant or synthetic nucleic acid molecules. Extreme caution shall be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Following use, needles shall not be bent, sheared, replaced in the needle sheath or guard or removed from the syringe. The needles and syringes shall be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix Q-II-C-1-g-(14). A biosafety manual shall be prepared or adopted. Personnel shall be advised of special hazards and required to read and follow instructions on practices and procedures.

Appendix Q-II-C-2. Animal Facilities (BL3-N)

Appendix Q-II-C-2-a. Animals shall be contained within an enclosed structure (animal room or equivalent) to minimize the possibility of theft or unintentional release and avoid arthropod access. The special provision to avoid the entry or escape of arthropods from the animal areas may be waived if the agent in use is not known to be transmitted by arthropods.

Appendix Q-II-C-2-b. The interior walls, floors, and ceilings shall be impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat, to facilitate cleaning. Penetrations in these structures and surfaces (e.g., plumbing and utilities) shall be sealed.

Appendix Q-II-C-2-c. Windows in the animal facility shall be closed, sealed, and breakage resistant (e.g., double-pane tempered glass or equivalent). The need to maintain negative pressure should be considered when constructing or renovating the animal facility.

Appendix Q-II-C-2-d. An autoclave, incinerator, or other effective means to decontaminate animals and waste shall be available, preferably within the containment area. If feasible, a double-door autoclave is preferred and should be positioned to allow removal of material from the containment area.

Appendix Q-II-C-2-e. If arthropods are used in the experiment or the agent under study can be transmitted by an arthropod, the interior work area shall be appropriately screened (52 mesh). All perimeter joints and openings shall be sealed, and additional arthropod control mechanisms used to minimize arthropod entry and propagation, including appropriate screening, or the equivalent of access doors.

Appendix Q-II-C-2-f. Access doors to the containment area shall be self-closing.

Appendix Q-II-C-2-g. The animal area shall be separated from all other areas. Passage through two sets of doors shall be the basic requirement for entry into the animal area from access corridors or other contiguous areas. The animal containment area shall be physically separated from access corridors and other laboratories or areas by a double-door clothes change room, equipped with integral showers and airlock.

Appendix Q-II-C-2-h. Liquid effluent from containment equipment, sinks, biological safety cabinets, animal rooms, primary barriers, floor drains, and sterilizers shall be decontaminated by heat treatment before being released into the sanitary system. The procedure used for heat decontamination of liquid wastes shall be monitored with a recording thermometer. The effectiveness of the heat decontamination process system shall be revalidated every 30 days with an indicator organism.

Appendix Q-II-C-2-i. An exhaust air ventilation system shall be provided. This system shall create directional airflow that draws air into the animal room through the entry area. The building exhaust, or the exhaust from primary containment units, may be used for this purpose if the exhaust air is discharged to the outside and shall be dispersed away from occupied areas and air intakes. Personnel shall verify that the direction of the airflow (into the animal room) is proper.

Appendix Q-II-C-2-j. If the agent is transmitted by aerosol, then the exhaust air shall pass through a high efficiency particulate air/HEPA filter.

Appendix Q-II-C-2-k. Vacuum lines shall be protected with high efficiency particulate air/HEPA filters and liquid disinfectant traps.

Appendix Q-II-C-2-l. In lieu of open housing in the special animal room, animals held in a BL3-N area may be housed in partial-containment caging systems (e.g., Horsfall units or gnotobiotic systems, or other special containment primary barriers). Prudent judgment must be exercised to implement this ventilation system (e.g., animal species) and its discharge location.

Appendix Q-II-C-2-m. Each animal area shall contain a foot, elbow, or automatically operated sink for hand washing. The sink shall be located near the exit door.

Appendix Q-II-C-2-n. Restraining devices for animals may be required to avoid damage to the integrity of the animal containment facility.

Appendix Q-II-D. Biosafety Level 4 - Animals (BL4-N) (See Appendix Q-III-C, *Footnotes and References for Appendix Q*)

Appendix Q-II-D-1. Standard Practices (BL4-N)

Appendix Q-II-D-1-a. Animal Facility Access (BL4-N)

Appendix Q-II-D-1-a-(1). Individuals under 16 years of age shall not be permitted to enter the animal area.

Appendix Q-II-D-1-a-(2). The containment area shall be locked.

Appendix Q-II-D-1-a-(3). The containment area shall be patrolled or monitored at frequent intervals.

Appendix Q-II-D-1-a-(4). The containment building shall be controlled and have a locking access.

Appendix Q-II-D-1-a-(5). The Animal Facility Director shall establish policies and procedures whereby only persons who have been advised of the potential hazard and who meet any specific entry requirements (e.g., vaccination) may enter the laboratory or animal room.

Appendix Q-II-D-1-a-(6). Individuals shall enter and exit the animal facility only through the clothing change and shower rooms.

Appendix Q-II-D-1-a-(7). Personnel shall use the airlocks to enter or exit the laboratory only in an emergency.

Appendix Q-II-D-1-a-(8). Animal room doors, gates, and other closures shall be kept closed when experiments are in progress.

Appendix Q-II-D-1-b. Decontamination and Inactivation (BL4-N)

Appendix Q-II-D-1-b-(1). All contaminated liquid or solid wastes shall be decontaminated before disposal.

Appendix Q-II-D-1-b-(2). The work surfaces and containment equipment shall be decontaminated when work with organisms containing recombinant or synthetic nucleic acid molecules is finished. Where feasible, plastic-backed paper toweling shall be used on nonporous work surfaces to facilitate clean-up.

Appendix Q-II-D-1-b-(3). All wastes from animal rooms and laboratories shall be appropriately decontaminated before disposal in an approved manner.

Appendix Q-II-D-1-b-(4). No materials, except for biological materials that are to remain in a viable or intact state, shall be removed from the maximum containment laboratory unless they have been autoclaved or decontaminated. Equipment or material that might be damaged by high temperatures or steam shall be decontaminated by gaseous or vapor methods in an airlock or chamber designed for this purpose.

Appendix Q-II-D-1-b-(5). When ventilated suits are required, the animal personnel shower entrance/exit area shall be equipped with a chemical disinfectant shower to decontaminate the surface of the suit before exiting the area. A neutralization or water dilution device shall be integral with the chemical disinfectant discharge piping before entering the heat sterilization system. Entry to this area shall be through an airlock fitted with airtight doors.

Appendix Q-II-D-1-b-(6). Needles and syringes shall be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix Q-II-D-1-b-(7). Supplies and materials needed in the animal facility shall be brought in by way of the double-door autoclave, fumigation chamber, or airlock that shall be appropriately decontaminated between each use.

Appendix Q-II-D-1-b-(8). An autoclave, incinerator, or other effective means to decontaminate animals and wastes shall be available, preferably within the containment area. If feasible, a double-door autoclave is preferred and should be positioned to allow removal of material from the containment area.

Appendix Q-II-D-1-b-(9). Liquid effluent from containment equipment, sinks, biological safety cabinets, animal rooms, primary barriers, floor drains, and sterilizers shall be decontaminated by heat treatment before being released into the sanitary system. Liquid wastes from shower rooms and toilets shall be decontaminated with chemical disinfectants or heat by methods demonstrated to be effective. The procedure used for heat decontamination of liquid wastes shall be monitored with a recording thermometer. The effectiveness of the heat decontamination process system shall be revalidated every 30 days with an indicator organism. Liquid wastes from the shower shall be chemically decontaminated using an Environmental Protection Agency-approved germicide. The efficacy of the chemical treatment process shall be validated with an indicator organism. Chemical disinfectants shall be neutralized or diluted before release into general effluent waste systems.

Appendix Q-II-D-1-c. Signs (BL4-N)

Appendix Q-II-D-1-c-(1). When the animal research requires special provisions for entry (e.g., vaccination), a warning sign incorporating the universal biosafety symbol shall be posted on all access doors to the animal work area. The sign shall indicate: (i) the agent, (ii) the animal species, (iii) the name and telephone number of the Animal Facility Director, or other responsible individual, and (iv) any special requirements for entering the laboratory.

Appendix Q-II-D-1-d. Protective Clothing (BL4-N)

Appendix Q-II-D-1-d-(1). Individuals shall enter and exit the animal facility only through the clothing change and shower rooms. Street clothing shall be removed and kept in the outer clothing change room. Complete laboratory clothing (may be disposable), including undergarments, pants, shirts, jump suits, and shoes shall be provided for all personnel entering the animal facility. When exiting the BL4-N area and before proceeding into the shower area, personnel shall remove their laboratory clothing in the inner change room. All laboratory clothing shall be autoclaved before laundering. Personnel shall shower each time they exit the animal facility.

Appendix Q-II-D-1-d-(2). A ventilated head-hood or a one-piece positive pressure suit, which is ventilated by a life-support system, shall be worn by all personnel entering rooms that contain experimental animals when appropriate. When ventilated suits are required, the animal personnel shower entrance/exit area shall be equipped with a chemical disinfectant shower to decontaminate the surface of the suit before exiting the area. A neutralization or water dilution device shall be integral with the chemical disinfectant discharge piping before entering the heat sterilization system. Entry to this area shall be through an airlock fitted with airtight doors.

Appendix Q-II-D-1-d-(3). Appropriate respiratory protection shall be worn in rooms containing experimental animals.

Appendix Q-II-D-1-e. Records (BL4-N)

Appendix Q-II-D-1-e-(1). Documents regarding experimental animal use and disposal shall be maintained in a permanent record book.

Appendix Q-II-D-1-e-(2). A system shall be established for: (i) reporting laboratory accidents and exposures that are a result of overt exposures to organisms containing recombinant or synthetic nucleic acid molecules, (ii) employee absenteeism, and (iii) medical surveillance of potential laboratory-associated illnesses. Permanent records shall be prepared and maintained. Any incident involving spills and accidents that results in environmental release or exposures of animals or laboratory workers to organisms containing recombinant or synthetic nucleic acid molecules shall be reported immediately to the Biological Safety Officer, Animal Facility Director, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to the NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment shall be provided as appropriate and written records maintained. If necessary, the area shall be appropriately decontaminated.

Appendix Q-II-D-1-e-(3). When appropriate and giving consideration to the agents handled, baseline serum samples shall be collected and stored for animal care and other at-risk personnel. Additional serum specimens may be collected periodically depending on the agents handled or the function of the facility.

Appendix Q-II-D-1-e-(4). A permanent record book indicating the date and time of each entry and exit shall be

signed by all personnel.

Appendix Q-II-D-1-f. Transfer of Materials (BL4-N)

Appendix Q-II-D-1-f-(1). No materials, except for biological materials that are to remain in a viable or intact state, shall be removed from the maximum containment laboratory unless they have been autoclaved or decontaminated. Equipment or material that might be damaged by high temperatures or steam shall be decontaminated by gaseous or vapor methods in an airlock or chamber designed for this purpose.

Appendix Q-II-D-1-f-(2). Biological materials removed from the animal maximum containment laboratory in a viable or intact state shall be transferred to a non-breakable sealed primary container and then enclosed in a non-breakable sealed secondary container that shall be removed from the animal facility through a disinfectant dunk tank, fumigation chamber, or an airlock designed for this purpose. Advance approval for transfer of material shall be obtained from the Animal Facility Director. Such packages containing viable agents can only be opened in another BL4-N animal facility if the agent is biologically inactivated or incapable of reproduction. Special safety testing, decontamination procedures, and Institutional Biosafety Committee approval shall be required to transfer agents or tissue/organ specimens from a BL4-N animal facility to one with a lower containment classification.

Appendix Q-II-D-1-f-(3). Supplies and materials needed in the animal facility shall be brought in by way of the double-door autoclave, fumigation chamber, or airlock that shall be appropriately decontaminated between each use. After securing the outer doors, personnel within the animal facility retrieve the materials by opening the interior doors of the autoclave, fumigation chamber, or airlock. These doors shall be secured after materials are brought into the animal facility.

Appendix Q-II-D-1-g. Other (BL4-N)

Appendix Q-II-D-1-g-(1). All genetically engineered neonates shall be permanently marked within 72 hours after birth, if their size permits. If their size does not permit marking, their containers should be marked. In addition, transgenic animals should contain distinct and biochemically assayable DNA sequences that allow identification of transgenic animals from among non-transgenic animals.

Appendix Q-II-D-1-g-(2). Eating, drinking, smoking, and applying cosmetics shall not be permitted in the work area.

Appendix Q-II-D-1-g-(3). Individuals who handle materials and animals containing recombinant or synthetic nucleic acid molecules shall be required to wash their hands before exiting the containment area.

Appendix Q-II-D-1-g-(4). Experiments involving other organisms that require containment levels lower than BL4-N may be conducted in the same area concurrently with experiments requiring BL4-N containment provided that they are conducted in accordance with BL4-N practices.

Appendix Q-II-D-1-g-(5). Animal holding areas shall be cleaned at least once a day and decontaminated immediately following any spill of viable materials.

Appendix Q-II-D-1-g-(6). All procedures shall be performed carefully to minimize the creation of aerosols.

Appendix Q-II-D-1-g-(7). A double barrier shall be provided to separate male and female animals. Animal isolation barriers shall be sturdy and accessible for cleaning. Reproductive incapacitation may be used.

Appendix Q-II-D-1-g-(8). The containment area shall be in accordance with state and Federal laws and animal care requirements.

Appendix Q-II-D-1-g-(9). The life support system for the ventilated suit or head hood is equipped with alarms and emergency back-up air tanks. The exhaust air from the suit area shall be filtered by two sets of high efficiency particulate air/HEPA filters installed in series or incinerated. A duplicate filtration unit, exhaust fan, and an automatically starting emergency power source shall be provided. The air pressure within the suit shall be greater than that of any adjacent area. Emergency lighting and communication systems shall be provided. A double-door autoclave shall be provided for decontamination of waste materials to be removed from the suit area.

Appendix Q-II-D-1-g-(10). Needles and syringes shall be used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) shall be used for the injection or aspiration of fluids containing organisms that contain recombinant or synthetic nucleic acid molecules. Extreme caution shall be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Following use, needles shall

not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe. The needles and syringes shall be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix Q-II-D-1-g-(11). An essential adjunct to the reporting-surveillance system is the availability of a facility for quarantine, isolation, and medical care of personnel with potential or known laboratory-associated illnesses.

Appendix Q-II-D-1-g-(12). A biosafety manual shall be prepared or adopted. Personnel shall be advised of special hazards and required to read and follow instructions on practices and procedures.

Appendix Q-II-D-1-g-(13). Vacuum lines shall be protected with high efficiency particulate air/HEPA filters and liquid disinfectant traps.

Appendix Q-II-D-2. Animal Facilities (BL4-N)

Appendix Q-II-D-2-a. Animals shall be contained within an enclosed structure (animal room or equivalent) to minimize the possibility of theft or unintentional release and avoid arthropod access.

Appendix Q-II-D-2-b. The interior walls, floors, and ceilings shall be impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat, to facilitate cleaning. Penetrations in these structures and surfaces (e.g., plumbing and utilities) shall be sealed.

Appendix Q-II-D-2-c. Windows in the animal facility shall be closed, sealed, and breakage resistant (e.g., double-pane tempered glass or equivalent).

Appendix Q-II-D-2-d. An autoclave, incinerator, or other effective means to decontaminate animals and wastes shall be available, preferably within the containment area. If feasible, a double-door autoclave is preferred and should be positioned to allow removal of material from the containment area.

Appendix Q-II-D-2-e. Access doors to the containment area shall be self-closing.

Appendix Q-II-D-2-f. All perimeter joints and openings shall be sealed to form an arthropod-proof structure.

Appendix Q-II-D-2-g. The BL4-N laboratory provides a double barrier to prevent the release of recombinant or synthetic nucleic acid molecule containing microorganisms into the environment. Design of the animal facility shall be such that if the barrier of the inner facility is breached, the outer barrier will prevent release into the environment. The animal area shall be separated from all other areas. Passage through two sets of doors shall be the basic requirement for entry into the animal area from access corridors or other contiguous areas. Physical separation of the animal containment area from access corridors or other laboratories or activities shall be provided by a double-door clothes change room equipped with integral showers and airlock.

Appendix Q-II-D-2-h. A necropsy room shall be provided within the BL4-N containment area.

Appendix Q-II-D-2-i. Liquid effluent from containment equipment, sinks, biological safety cabinets, animal rooms, primary barriers, floor drains, and sterilizers shall be decontaminated by heat treatment before being released into the sanitary system. Liquid wastes from shower rooms and toilets shall be decontaminated with chemical disinfectants or heat by methods demonstrated to be effective. The procedure used for heat decontamination of liquid wastes shall be monitored with a recording thermometer. The effectiveness of the heat decontamination process system shall be revalidated every 30 days with an indicator organism. Liquid wastes from the shower shall be chemically decontaminated using an Environmental Protection Agency-approved germicide. The efficacy of the chemical treatment process shall be validated with an indicator

organism. Chemical disinfectants shall be neutralized or diluted before release into general effluent waste systems.

Appendix Q-II-D-2-j. A ducted exhaust air ventilation system shall be provided that creates directional airflow that draws air into the laboratory through the entry area. The exhaust air, which is not recirculated to any other area of the building, shall be discharged to the outside and dispersed away from the occupied areas and air intakes. Personnel shall verify that the direction of the airflow (into the animal room) is proper.

Appendix Q-II-D-2-k. Exhaust air from BL4-N containment area shall be double high efficiency particulate air/HEPA filtered or treated by passing through a certified HEPA filter and an air incinerator before release to the atmosphere. Double HEPA filters shall be required for the supply air system in a BL4-N containment area.

Appendix Q-II-D-2-l. All high efficiency particulate air/HEPA filters' frames and housings shall be certified to have no detectable smoke [dioctylphthalate] leaks when the exit face (direction of flow) of the filter is scanned above 0.01 percent when measured by a linear or logarithmic photometer. The instrument must demonstrate a threshold sensitivity of at least $1x10^{-3}$ micrograms per liter for 0.3 micrometer diameter dioctylphthalate particles and a challenge concentration of 80-120 micrograms per liter. The air sampling rate should be at least 1 cfm (28.3 liters per minute).

Appendix Q-II-D-2-m. If an air incinerator is used in lieu of the second high efficiency particulate air/HEPA filter, it shall be biologically challenged to prove all viable test agents are sterilized. The biological challenge must be minimally $1x10^{8}$ organisms per cubic foot of airflow through the incinerator. It is universally accepted if bacterial spores are used to challenge and verify that the equipment is capable of killing spores, then assurance is provided that all other known agents are inactivated by the parameters established to operate the equipment. Test spores meeting this criterion are *Bacillus subtilis* var. *niger* or *Bacillus stearothermophilis*. The operating temperature of the incinerator shall be continuously monitored and recorded during use.

Appendix Q-II-D-2-n. All equipment and floor drains shall be equipped with deep traps (minimally 5 inches). Floor drains shall be fitted with isolation plugs or fitted with automatic water fill devices.

Appendix Q-II-D-2-o. Each animal area shall contain a foot, elbow, or automatically operated sink for hand washing. The sink shall be located near the exit door.

Appendix Q-II-D-2-p. Restraining devices for animals may be required to avoid damage to the integrity of the containment animal facility.

Appendix Q-II-D-2-q. The supply water distribution system shall be fitted with a back-flow preventer or break tank.

Appendix Q-II-D-2-r. All utilities, liquid and gas services, shall be protected with devices that avoid back-flow.

Appendix Q-II-D-2-s. Sewer and other atmospheric ventilation lines shall be equipped minimally with a single high efficiency particulate/HEPA filter. Condensate drains from these type housings shall be appropriately connected to a contaminated or sanitary drain system. The drain position in the housing dictates the appropriate system to be used.

Appendix Q-III. Footnotes and References for Appendix Q

Appendix Q-III-A. If a recombinant or synthetic nucleic acid molecule is derived from a Class 2 organism requiring BL2 containment, personnel shall be required to have specific training in handling pathogenic agents and directed by knowledgeable scientists.

Appendix Q-III-B. Personnel who handle pathogenic and potentially lethal agents shall be required to have specific training and be supervised by knowledgeable scientists who are experienced in working with these agents. BL3-N containment also minimizes escape of recombinant or synthetic nucleic acid molecule-containing organisms from exhaust air or waste material from the containment area.

Appendix Q-III-C. Risk Group 4 and restricted microorganisms (see Appendix B, *Classification of Human Etiologic Agents on the Basis of Hazard*, and Sections V-G and V-L, *Footnotes and References of Sections I through IV*) pose a high level of individual risk for acquiring life-threatening diseases to personnel and/or

animals. To import animal or plant pathogens, special approval must be obtained from U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Veterinary Services, National Center for Import-Export, Products Program, 4700 River Road, Unit 40, Riverdale, MD 20737. Phone: (301) 734-8499; Fax: (301) 734-8226.

Laboratory staff shall be required to have specific and thorough training in handling extremely hazardous infectious agents, primary and secondary containment, standard and special practices, and laboratory design characteristics. The laboratory staff shall be supervised by knowledgeable scientists who are trained and experienced in working with these agents and in the special containment facilities.

Within work areas of the animal facility, all activities shall be confined to the specially equipped animal rooms or support areas. The maximum animal containment area and support areas shall have special engineering and design features to prevent the dissemination of microorganisms into the environment via exhaust air or waste disposal.

Appendix Q-III-D. Other research with non-laboratory animals, which may not appropriately be conducted under conditions described in Appendix Q, may be conducted safely by applying practices routinely used for controlled culture of these biota. In aquatic systems, for example, BL1 equivalent conditions could be met by utilizing growth tanks that provide adequate physical means to avoid the escape of the aquatic species, its gametes, and introduced exogenous genetic material. A mechanism shall be provided to ensure that neither the organisms nor their gametes can escape into the supply or discharge system of the rearing container (e.g., tank, aquarium, etc.) Acceptable barriers include appropriate filtration, irradiation, heat treatment, chemical treatment, etc. Moreover, the top of the rearing container shall be covered to avoid escape of the organism and its gametes. In the event of tank rupture, leakage, or overflow, the construction of the room containing these tanks should prevent the organisms and gametes from entering the building's drains before the organism and its gametes have been inactivated.

Other types of non-laboratory animals (e.g., nematodes, arthropods, and certain forms of smaller animals) may be accommodated by using the appropriate BL1 through BL4 or BL1-P through BL4-P containment practices and procedures as specified in Appendices G and P.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES

Guidelines for Research Involving Recombinant DNA Molecules

May 1986.

These NIH Guidelines supersede earlier versions and will be in effect until further notice.

Table of Contents

I. Scope of the Guidelines

I–A—Purpose

The purpose of these Guidelines is to specify practices for constructing and handling (i) recombinant DNA molecules and (ii) organisms and viruses containing recombinant DNA molecules.

I–B—Definition of Recombinant DNA Molecules

In the context of these Guidelines, recombinant DNA molecules are defined as either (i) molecules which are constructed outside living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell, or (ii) DNA molecules that result from the replication of those described in (i) above.

Synthetic DNA segments likely to yield a potentially harmful polynucleotide or polypeptide (e.g., a toxin or a pharmocologically active agent) shall be considered as equivalent to their natural DNA counterpart. If the synthetic DNA segment is not expressed *in vivo* as a biologically active polynucleotide or polypeptide product, it is exempt from the Guidelines.

I–C—General Applicability

The Guidelines are applicable to all recombinant DNA research within the United States or its territories which is conducted at or sponsored by an institution that receives any support for recombinant DNA research from the National Institutes of Health (NIH). This includes research performed by NIH directly.

An individual receiving support for research involving recombinant DNA must be associated with or sponsored by an institution that can and does assume the responsibilities assigned in these Guidelines.

The Guidelines are also applicable to projects done abroad if they are supported by NIH funds. If the host country, however, has established rules for the conduct of recombinant DNA projects, then a certificate of compliance with those rules may be submitted to NIH in lieu of compliance with the NIH Guidelines. The NIH reserves the right to withhold funding if the safety practices to be employed abroad are not reasonably consistent with the NIH Guidelines.

I–D—General Definitions

The following terms, which are used throughout the Guidelines, are defined as follows:

I–D–1. "Institution" means any public or private entity (including Federal, State, and local government agencies).

I–D–2. "Institutional Biosafety Committee" or "IBC" means a committee that (i) meets the requirements for membership specified in Section IV–B–2, and (ii) reviews, approves, and oversees projects in accordance with the responsibilities defined in Sections IV–B–2 and IV–B–3.

I–D–3. "NIH Office of Recombinant DNA Activities" or "ORDA" means the office within NIH with responsibility for (i) reviewing and coordinating all activities of NIH related to the Guidelines, and (ii) performing other duties as defined in Section IV–C–3.

I–D–4. "Recombinant DNA Advisory Committee" of "RAC" means the public advisory committee that advises the Secretary, the Assistant Secretary for Health, and the Director, NIH, concerning recombinant DNA research. The RAC shall be constituted as specified in Section IV–C–2.

I–D–5. "Director, NIH" or "Director" means the Director, NIH, or any other officer or employee of NIH to whom authority has been delegated.

II. Containment

Effective biological safety programs have been operative in a variety of laboratories for many years. Considerable information, therefore, already exists for the design of physical containment facilities and the selection of laboratory procedures applicable to organisms carrying recombinant DNAs [3–16]. The existing programs rely upon mechanisms that, for convenience, can be divided into two categories: (i) A set of standard practices that are generally used in microbiological laboratories; and (ii) special procedures, equipment, and laboratory installations that provide physical barriers which are applied in varying degrees according to the estimated biohazard. Four biosafety levels (BL) are described in Appendix G. These biosafety levels consist of combinations of laboratory practices and techniques, safety equipment, and laboratory facilities appropriate for the operations performed and the hazard posed by agents and for the laboratory function and activity. Biosafety level 4 (BL4) provides the most stringent containment conditions, BL1 the least stringent.

Experiments on recombinant DNAs by their very nature lend themselves to a third containment mechanism—namely, the application of highly specific biological barriers. In fact, natural barriers do exist which limit either (i) the infectivity of a *vector* or *vehicle* (plasmid or virus) for specific hosts, or (ii) its dissemination and survival in the environment. The vectors that provide the means for replication of the recombinant DNAs and/or the host cells in which they replicate can be genetically designed to decrease by many orders of magnitude the probability of dissemination of recombinant DNAs outside the laboratory. Further details on biological containment may be found in Appendix I.

As these three means of containment are complementary, different levels of containment appropriate for experiments with different recombinants can be established by applying various combinations of the physical and biological barriers along with a constant use of the standard practices. We consider these categories of containment separately in order that such combinations can be conveniently expressed in the Guidelines.

In constructing these Guidelines, it was necessary to define boundary conditions for the different levels of physical and biological containment and for the classes of experiments to which they apply. We recognize that these definitions do not take into account all existing and anticipated information on special procedures that will allow particular experiments to be carried out under different conditions than indicated here without affecting risk. Indeed, we urge that individual investigators devise simple and more effective containment procedures, and that investigators and IBCs recommend changes in the Guidelines to permit their use.

III. Guidelines for Covered Experiments

Part III discusses experiments involving recombinant DNA. These experiments have been divided into four classes:

III–A. Experiments which require specific RAC review and NIH and IBC approval before initiation of the experiment;

III–B. Experiments which require IBC approval before initiation of the experiment;

III–C. Experiments which require IBC notification at the time of initiation of the experiment;

III–D. Experiments which are exempt from the procedures of the Guidelines.

IF AN EXPERIMENT FALLS INTO BOTH CLASS III–A AND ONE OF THE OTHER CLASSES, THE RULES PERTAINING TO CLASS III–A MUST BE FOLLOWED. If an experiment falls into Class III–D and into either Class III–B or III–C as well, it can be considered

exempt from the requirements of the Guidelines.

Changes in containment levels from those specified here may not be instituted without the express approval of the Director, NIH (see Sections IV-C-1-b-(1), IV-C-1-b-(2), and subsections).

III-A—Experiments That Require RAC Review and NIH and IBC Approval Before Initiation

Experiments in this category cannot be initiated without submission of relevant information on the proposed experiment to NIH, the publication of the proposal in the **Federal Register** for thirty days of comment, review by the RAC, and specific approval by NIH. The containment conditions for such experiments will be recommended by RAC and set by NIH at the time of approval. Such experiments also require the approval of the IBC before initiation. Specific experiments already approved in this section and the appropriate containment conditions are listed in Appendices D and F. If an experiment is similar to those listed in Appendices D and F, ORDA may determine appropriate containment conditions according to case precedents under Section IV-C-1-b-(3)-(g).

If the experiments in this category are submitted for review to another Federal agency, the submitter shall notify ORDA; ORDA may then determine that such review serves the same purpose, and based on that determination, notify the submitter that no RAC review will take place, no NIH approval is necessary, and the experiment may proceed upon approval from the other Federal agency.

III-A-1. Deliberate formation of recombinant DNAs containing genes for the biosynthesis of toxic molecules lethal for vertebrates at an LD_{50} of less than 100 nanograms per kilogram body weight (e.g., microbial toxins such as the botulinum toxins, tetanus toxin, diphtheria toxin, *Shigella dysenteriae* neurotoxin). Specific approval has been given for the cloning in *E. coli* K-12 of DNAs containing genes coding for the biosynthesis of toxic molecules which are lethal to vertebrates at 100 nanograms to 100 micrograms per kilogram body weight. Containment levels for these experiments are specified in Appendix F.

III-A-2. Deliberate release into the environment of any organism containing recombinant DNA, except certain plants as described in Appendix L.

III-A-3. Deliberate transfer of a drug resistance trait to microorganisms that are not known to acquire it naturally [2], if such acquisition could compromise the use of the drug to control disease agents in human or veterinary medicine or agriculture.

III-A-4. Deliberate transfer of recombinant DNA or DNA or RNA derived from recombinant DNA into human subjects [21]. The requirement for RAC review should not be considered to preempt any other required review of experiments with human subjects. Institutional Review Board (IRB) review of the proposal should be completed before submissin to NIH.

III-B—Experiments That Require IBC Approval Before Initiation

Investigators performing experiments in this category must submit to their IBC, prior to initiation of the experiments, a registration document that contains a description of: (i) The source(s) of DNA; (ii) the nature of the inserted DNA sequences; (iii) the hosts and vectors to be used; (iv) whether a deliberate attempt will be made to obtain expression of a foreign gene, and, if so, what protein will be produced; and (v) the containment conditions specified in these Guidelines. This registration document must be dated and signed by the investigator and filed only with the local IBC. The IBC shall review all such proposals prior to initiation of the experiments. Requests for lowering of containment for experiments in this category will be considered by NIH (see Section IV-C-1-b-(3)).

III-B-1—Experiments Using Human or Animal Pathogens (Class 2, Class 3, Class 4, or Class 5 Agents [1]) as Host-Vector Systems

III-B-1-a. Experiments involving the introduction of recombinant DNA into Class 2 agents can be carried out at BL2 containment.

III-B-1-b. Experiments involving the introduction of recombinant DNA into Class 3 agents can be carried out at BL3 containment.

III-B-1-c. Experiments involving the introduction of recombinant DNA into Class 4 agents can be carried out at BL4 containment.

III-B-1-d. Containment conditions for experiments involving the introduction of recombinant DNA into Class 5 agents will be set on a case-by-case basis following ORDA review. A U.S. Department of Agriculture (USDA) permit is required for work with Class 5 agents [18, 20].

III-B-2—Experiments in Which DNA From Human or Animal Pathogens (Class 2, Class 3, Class 4, or Class 5 Agents [1]) is Cloned in Nonpathogenic Prokaryotic or Lower Eukaryotic Host-Vector Systems

III-B-2-a. Recombinant DNA experiments in which DNA from Class 2 or Class 3 agents [1] is transferred into nonpathogenic prokaryotes or lower eukaryotes may be performed under BL2 containment. Recombinant DNA experiments in which DNA from Class 4 agents is transferred into nonpathogenic prokaryotes or lower eukaryotes can be performed at BL2 containment after demonstration that only a totally and irreversibly defective fraction of the agent's genome is present in a given recombinant. In the absence of such a demonstration, BL4 containment should be used. Specific lowering of containment of BL1 for particular experiments can be approved by the IBC. Many experiments in this category will be exempt from the Guidelines (see Sections III-D-4 and III-D-5). Experiments involving the formation of recombinant DNAs for certain genes coding for molecules toxic for vertebrates require RAC review and NIH approval (see Section III-A-1) or must be carried out under NIH specified conditions as described in Appendix F.

III-B-2-b. Containment conditions for experiments in which DNA from Class 5 agents is transferred into nonpathogenic prokaryotes or lower eukaryotes will be determined by ORDA following a case-by-case review. A USDA permit is required for work with Class 5 agents [18, 20].

III-B-3—Experiments Involving the Use of Infectious Animal or Plant DNA or RNA Viruses or Defective Animal or Plant DNA or RNA Viruses in the Presence of Helper Virus in Tissue Culture Systems

Caution: Special care should be used in the evaluation of containment levels for experiments which are likely to either enhance the pathogenicity (e.g., insertion of a host oncogene) or to extend the host range (e.g., introduction of novel control elements) of viral vectors under conditions which permit a productive infection. In such cases, serious consideration should be given to raising the physical containment by at least one level.

Note.—Recombinant DNA molecules or RNA molecules derived therefrom, which contain less than two-thirds of the genome of any eukaryotic virus (all virus from a single Family [17] being considered identical [19]), may be considered defective and can be used

in the absence of helper under the conditions specified in Section III-C.

III-B-3-a. Experiments involving the use of infectious Class 2 animal viruses [1] or defective Class 2 animal viruses in the presence of helper virus can be performed at BL2 containment.

III-B-3-b. Experiments involving the use of infectious Class 3 animal viruses [1] or defective Class 3 animal viruses in the presence of helper virus can be carried out at BL3 containment.

III-B-3-c. Experiments involving the use of infectious Class 4 viruses [1] or defective Class 4 viruses in the presence of helper virus may be carried out under BL4 containment.

III-B-3-d. Experiments involving the use of infectious Class 5 [1] viruses or defective Class 5 viruses in the presence of helper virus will be determined on a case-by-case basis following ORDA review. A USDA permit is required for work with Class 5 pathogens [18, 20].

III-B-3-e. Experiments involving the use of infectious animal or plant viruses or defective animal or plant viruses in the presence of helper virus not covered by Sections III-B-3-a, III-B-3-b, III-B-3-c, or III-B-3-d may be carried out under BL1 containment.

III-B-4—Recombinant DNA Experiments Involving Whole Animals or Plants

III-B-4-a. Recombinant DNA, or RNA molecules derived therefrom, from any source except for greater than two-thirds of a eukaryotic viral genome may be transferred to any non-human vertebrate organism and propagated under conditions of physical containment comparable to BL1 and appropriate to the organism under study [2]. It is important that the investigator demonstrate that the fraction of the viral genome being utilized does not lead to productive infection. A USDA permit is required for work with Class 5 agents [18, 20].

III-B-4-b. For all experiments involving whole animals and plants and not covered by Section III-B-4-a, the appropriate containment will be determined by the IBC [22].

III-B-5—Experiments Involving More Than 10 Liters of Culture

The appropriate containment will be decided by the IBC. Where appropriate, Appendix K, *Physical Containment for Large-Scale Uses of Organisms Containing Recombinant DNA Molecules*, should be used.

III-C. Experiments That Require IBC Notice Simultaneously With Initiation of Experiments

Experiments not included in Sections III-A, III-B, III-D, and subsections of these sections are to be considered in Section III-C. All such experiments can be carried out at BL1 containment. For experiments in this category, a registration document as described in Section III-B must be dated and signed by the investigator and filed with the local IBC at the time of initiation of the experiment. The IBC shall review all such proposals, but IBC review prior to initiation of the experiment is not required. [The reader should refer to the policy statement in the first two paragraphs of Section IV-A.)

For example, experiments in which all components derive from non-pathogenic prokaryotes and non-pathogenic lower eukaryotes fall under Section III-C and can be carried out at BL1 containment.

CAUTION: Experiments Involving Formation of Recombinant DNA Molecules Containing no more than Two-Thirds of the Genome of any Eukaryotic Virus. Recombinant DNA molecules containing no more than two-thirds of the genome of any eukaryotic virus (all viruses from a single Family [17] being considered identical [19]) may be propagated and maintained in cells in tissue culture using BL1 containment. For such experiments, it must be shown that the cells lack helper virus for the specific Families of defective viruses being used. If helper virus is present, procedures specified under Section III-B-3 should be used. The DNA may contain fragments of the genome of viruses from more than one Family but each fragment must be less than two-thirds of a genome.

III-D—Exempt Experiments

The following recombinant DNA molecules are exempt from these Guidelines and no registration with the IBC is necessary:

III-D-1. Those that are not in organisms or viruses.

III-D-2. Those that consist entirely of DNA segments from a single nonchromosomal or viral DNA source though one or more of the segments may be a synthetic equivalent.

III-D-3. Those that consist entirely of DNA from a prokaryotic host including its indigenous plasmids or viruses when propagated only in that host (or a closely related strain of the same species) or when transferred to another host by well established physiological means; also, those that consist entirely of DNA from an eukaryotic host including its chloroplasts, mitochondria, or plasmids (but excluding viruses) when propagated only in that host (or a closely related strain of the same species).

III-D-4. Certain specified recombinant DNA molecules that consist entirely of DNA segments from different species that exchange DNA by known physiological processes though one or more of the segments may be a synthetic equivalent. A list of such exchangers will be prepared and periodically revised by the Director, NIH, with advice of the RAC after appropriate notice and opportunity for public comment (see Section IV-C-1-b-(1)-(c)). Certain classes are exempt as of publication of these revised Guidelines. This list is in Appendix A. An updated list may be obtained from the Office of Recombinant DNA Activities, National Institutes of Health, Building 31, Room 3B10, Bethesda, Maryland 20892.

III-D-5. Other classes of recombinant DNA molecules—if the Director, NIH, with advice of the RAC, after appropriate notice and opportunity for public comment, finds that they do not present a significant risk to health or the environment (see Section IV-C-1-b-(1)-(c)). Certain classes are exempt as of publication of these revised Guidelines. The list is in Appendix C. An updated list may be obtained from the Office of Recombinant DNA Activities, National Institutes of Health, Building 31, Room 3B10, Bethesda, Maryland 20892.

IV. Roles and Responsibilities

IV-A—Policy

Safety in activities involving recombinant DNA depends on the individual conducting them. The Guidelines cannot anticipate every possible situation. Motivation and good judgment are the key essentials to protection of health and the environment.

The Guidelines are intended to help the institution, Institutional Biosafety Committee (IBC), Biological Safety Officer (BSO), and Principal Investigator (PI) determine the safeguards that should be implemented. These Guidelines will never be complete or final, since all conceivable experiments involving recombinant DNA cannot be foreseen. Therefore, *it is the responsibility of the institution and those associated with it to adhere to the intent of the Guidelines as well as to their specifics.*

Each institution (and the IBC acting on its behalf) is responsible for ensuring that recombinant DNA activities comply with the Guidelines. General recognition of institutional authority and

responsibility properly establishes accountability for safe conduct of the research at the local level.

The following roles and responsibilities constitute an administrative framework in which safety is an essential and integral part of research involving recombinant DNA molecules. Further clarifications and interpretations of roles and responsibilities will be issued by NIH as necessary.

IV–B—Responsibility of the Institution

IV–B–1. General Information. Each institution conducting or sponsoring recombinant DNA research covered by these Guidelines is responsible for ensuring that the research is carried out in full conformity with the provisions of the Guidelines. In order to fulfill this responsibility, the institution shall:

IV–B–1–a. Establish and implement policies that provide for the safe conduct of recombinant DNA research and that ensure compliance with the Guidelines. The institution as part of its general responsibilities for implementing the Guidelines may establish additional procedures as deemed necessary to govern the institution and its components in the discharge of its responsibilities under the Guidelines. This may include: (i) Statements formulated by the institution for general implementation of the Guidelines, and (ii) whatever additional precautionary steps the institution may deem appropriate.

IV–B–1–b. Establish an IBC that meets the requirements set forth in Section IV–B–2 and carries out the functions detailed in Section IV–B–3.

IV–B–1–c. If the institution is engaged in recombinant DNA research at the BL3 or BL4 containment level, appoint a BSO, who shall be a member of the IBC and carry out the duties specified in Section IV–B–4.

IV–B–1–d. Require that investigators responsible for research covered by these Guidelines comply with the provisions of Section IV–B–5 and assist investigators to do so.

IV–B–1–e. Ensure appropriate training for the IBC chairperson and members, the BSO, PIs, and laboratory staff regarding the Guidelines, their implementation, and laboratory safety. Responsibility for training IBC members may be carried out through the IBC chairperson. Responsibility for training laboratory staff may be carried out through the PI. The institution is responsible for seeing that the PI has sufficient training but may delegate this responsibility to the IBC.

IV–B–1–f. Determine the necessity in connection with each project for health surveillance of recombinant DNA research personnel, and conduct, if found appropriate, a health surveillance program for the project. [The "Laboratory Safety Monograph" (LSM) discusses various possible components of such a program—for example, records of agents handled, active investigation of relevant illnesses, and the maintenance of serial serum samples for monitoring serologic changes that may result from the employees' work experience. Certain medical conditions may place a laboratory worker at increased risk in any endeavor where infectious agents are handled. Examples given in the LSM include gastrointestinal disorders and treatment with steroids, immunosuppressive drugs, or antibiotics. Workers with such disorders or treatment should be evaluated to determine whether they should be engaged in research with potentially hazardous organisms during their treatment or illness. Copies of the LSM are available from ORDA.]

IV–B–1–g. Report within 30 days to ORDA any significant problems with and violations of the Guildelines and significant research-related accidents and illnesses, unless the institution determines that the PI or IBC has done so.

IV–B–2. Membership and Procedures of the IBC. The institution shall establish an IBC whose responsibilities need not be restricted to recombinant DNA. The committee shall meet the following requirements:

IV–B–2–a. The IBC shall comprise no fewer than five members so selected that they collectively have experience and expertise in recombinant DNA technology and the capability to assess the safety of recombinant DNA research experiments and any potential risk to public health or the environment. At least two members shall not be affiliated with the institution (apart from their membership on the IBC) and shall represent the interest of the surrounding community with respect to health and protection of the environment. Members meet this requirement if, for example, they are officials of State or local public health or environmental protection agencies, members of other local governmental bodies, or persons active in medical, occupational health, or environmental concerns in the community. The BSO, mandatory when research is being conducted at the BL3 and BL4 levels, shall be a member (see Section IV–B–4).

IV–B–2–b. In order to ensure the competence necessary to review recombinant DNA activities, it is recommended that: (i) The IBC include persons with expertise in recombinant DNA technology, biological safety, and physical containment; (ii) the IBC include, or have available as consultants, persons knowledgeable in institutional commitments and policies, applicable law, standards of professional conduct and practice, community attitudes, and the environment; and (iii) at least one member be from the laboratory technical staff.

IV–B–2–c. The institution shall identify the committee members by name in a report to ORDA and shall include relevant background information on each member in such form and at such times as ORDA may require.

IV–B–2–d. No member of an IBC may be involved (except to provide information requested by the IBC) in the review or approval of a project in which he or she has been or expects to be engaged or has a direct financial interest.

IV–B–2–e. The institution, who is ultimately responsible for the effectiveness of the IBC, may establish procedures that the IBC will follow in its initial and continuing review of applications, proposals, and activities. (IBC review procedures are specified in Section IV–B–3–a.)

IV–B–2–f. Institutions are encouraged to open IBC meetings to public whenever possible, consistent with protection of privacy and proprietary interests.

IV–B–2–g. Upon request, the institution shall make available to the public all minutes of IBC meetings and any documents submitted to or received from funding agencies which the latter are required to make available to the public. If comments are made by members of the public on IBC actions, the institution shall forward to NIH both the comments and the ICB's response.

IV–B–3. Functions of the IBC. On behalf of the institution, the IBC is responsible for:

IV–B–3–a. Reviewing for compliance with the NIH Guidelines recombinant DNA research as specified in Part III conducted at or sponsored by the institution, and approving those research projects that it finds are in conformity with the Guidelines. This review shall include:

IV–B–3–a–(1). An independent assessment of the containment levels required by these Guidelines for the proposed research, and

IV–B–3–a–(2). An assessment of the facilities, procedures, and practices, and of the training and expertise of recombinant DNA personnel.

IV–B–3–b. Notifying the PI of the results of their review.

IV–B–3–c. Lowering containment levels for certain experiments as specified in Sections III–B–2.

IV–B–3–d. Setting containment levels as specified in Section III–B–4–b and III–B–5.

IV–B–3–e. Reviewing periodically recombinant DNA research being conducted at the institution to ensure that the requirements of the Guidelines are being fulfilled.

IV–B–3–f. Adopting emergency plans covering accidental spills and personnel contamination resulting from such research.

Note.—Basic elements in developing specific procedures for dealing with major spills of potentially hazardous materials in the laboratory are detailed in the LSM. Included are information and references on decontamination and emergency plans. The NIH and the Centers for Disease Control are available to provide consultation and direct assistance, if necessary, as posted in the LSM. The institution shall cooperate with the State and local public health departments reporting any significant research-related illness or accident that appears to be a hazard to the public health.

IV–B–3–g. Reporting within 30 days to the appropriate institutional official and to ORDA any significant problems with or violations of the Guidelines and any significant research-related accidents or illnesses unless the IBC determines that the PI has done so.

IV–B–3–h. The IBC may not authorize initiation of experiments not explicitly covered by the Guidelines until NIH (with the advice of the RAC when required) establishes the containment requirement.

IV–B–3–i. Performing such other functions as may be delegated to the IBC under Section IV–B–1.

IV–B–4. Biological Safety Officer. The institution shall appoint a BSO if it engages in recombinant DNA research at the BL3 or BL4 containment level. The officer shall be a member of the IBC, and his or her duties shall include (but need not be limited to):

IV–B–4–a. Ensuring through periodic inspections that laboratory standards are rigorously followed;

IV–B–4–b. Reporting to the IBC and the institution all significant problems with and violations of the Guidelines and all significant research-related accidents and illnesses of which the BSO becomes aware unless the BSO determines that the PI has done so;

IV–B–4–c. Developing emergency plans for dealing with accidental spills and personnel contamination and investigating recombinant DNA research laboratory accidents;

IV–B–4–d. Providing advice on laboratory security;

IV–B–4–e. Providing technical advice to the PI and the IBC on research safety procedures.

Note.—See the LSM for additional information on the duties of the BSO.

IV–B–5. Principal Investigator (PI). On behalf of the institution, the PI is responsible for complying fully with the Guidelines in conducting any recombinant DNA research.

IV–B–5. PI—General. As part of this general responsibility, the PI shall:

IV–B–5–a–(1). Initiate or modify no recombinant DNA research requiring approval by the IBC prior to initiation (see Sections III–A and III–B) until that research or the proposed modification thereof has been approved by the IBC and has met all other requirements of the Guidelines;

IV–B–5–a–(2). Determine whether experiments are covered by Section III–C and follow the appropriate procedures;

IV–B–5–a–(3). Report within 30 days to the IBC and NIH (ORDA) all significant problems with and violations of the Guidelines and all significant research-related accidents and illnesses;

IV–B–5–a–(4). Report to the IBC and to NIH (ORDA) new information bearing on the Guidelines;

IV–B–5–a–(5). Be adequately trained in good microbiological techniques;

IV–B–5–a–(6). Adhere to IBC-approved emergency plans for dealing with accidental spills and personnel contamination; and

IV–B–5–a–(7). Comply with shipping requirements for recombinant DNA molecules. (See Appendix H for shipping requirements and the LSM for technical recommendations.)

IV–B–5–b. Submissions by the PI to NIH. The PI shall:

IV–B–5–b–(1). Submit information to NIH (ORDA) in order to have new host-vector systems certified;

IV–B–5–b–(2). Petition NIH with notice to the IBC for exemptions to these Guidelines;

IV–B–5–b–(3). Petition NIH with concurrence of the IBC for approval to conduct experiments specified in Section III–A of the Guidelines;

IV–B–5–b–(4). Petition NIH for determination of containment for experiments requiring case-by-case review;

IV–B–5–b–(5). Petition NIH for determination of containment for experiments not covered by the Guidelines.

IV–B–5–c. Submissions by the PI to the IBC. The PI shall:

IV–B–5–c–(1). Make the initial determination of the required levels of physical and biological containment in accordance with the Guidelines;

IV–B–5–c–(2). Select appropriate microbiological practices and laboratory techniques to be used in the research;

IV–B–5–c–(3). Submit the initial research protocol if covered under Guidelines Section III–A, III–B, or III–C (and also subsequent changes—e.g., changes in the source of DNA or host-vector system) to the IBC for review and approval or disapproval; and

IV–B–5–c–(4). Remain in communication with the IBC throughout the conduct of the project.

IV–B–5–d. PI Responsibilities Prior to Initiating Research. The PI is responsible for:

IV–B–5–d–(1). Making available to the laboratory staff copies of the protocols that describe the potential biohazards and the precautions to be taken;

IV–B–5–d–(2). Instructing and training staff in the practices and techniques required to ensure safety and in the procedures for dealing with accidents; and

IV–B–5–d–(3). Informing the staff of the reasons and provisions for any precautionary medical practices advised or requested, such as vaccinations or serum collection.

IV–B–5–e. PI Responsibilities During the Conduct of the Research. The PI is responsible for:

IV–B–5–e–(1). Supervising the safety performance of the staff to ensure that the required safety practices and techniques are employed;

IV–B–5–e–(2). Investigating and reporting in writing to ORDA, the BSO (where applicable), and the IBC any significant problems pertaining to the operation and implementation of containment practices and procedures;

IV–B–5–e–(3). Correcting work errors and conditions that may result in the release of recombinant DNA materials;

IV–B–5–e–(4). Ensuring the integrity of the physical containment (e.g., biological safety cabinets) and the biological containment (e.g., purity and genotypic and phenotypic characteristics).

IV–C—Responsibilities of NIH

IV–C–1. Director. The Director, NIH, is responsible for (i) establishing the NIH Guidelines for Research Involving Recombinant DNA Molecules, (ii) overseeing their implementation, and (iii) their final interpretation.

The Director has responsibilities under the Guidelines that involve ORDA and RAC. The ORDA's responsibilities under the Guidelines are administrative. Advice from the RAC is primarily

scientific and technical. In certain circumstances, there is specific opportunity for public comment with published response before final action.

IV–C–1–a. General Responsibilities of the Director, NIH. The responsibilities of the director shall include the following:

IV–C–1–a–(1). Promulgating requirements as necessary to implement the Guidelines;

IV–C–1–a–(2). Establishing and maintaining the RAC to carry out the responsibilities set forth in Section IV–C–2. The RAC's membership is specified in its charter and in Section IV–C–2;

IV–C–1–a–(3). Establishing and maintaining ORDA to carry out the responsibilities defined in Section IV–C–3.

IV–C–1–b. Specific Responsibilities of the Director, NIH. In carrying out the responsibilities set forth in this section, the director or a designee shall weigh each proposed action through appropriate analysis and consultation to determine that it complies with the Guidelines and presents no significant risk to health or the environment.

IV–C–1–b–(1). Major Actions. To execute major actions the director must seek the advice of the RAC and provide an opportunity for public and Federal agency comment. Specifically, the agenda of the RAC meeting citing the major actions will be published in the **Federal Register** at least 30 days before the meeting, and the director will also publish the proposed actions in the **Federal Register** for comment as least 30 days before the meeting. In addition, the director's proposed decision, at his discretion, may be published in the **Federal Register** for 30 days of comment before final action is taken. The director's final decision, along with response to the comments, will be published in the **Federal Register** and the *Recombinant DNA Technical Bulletin.* The RAC and IBC chairpersons will be notified of this decision:

IV–C–1–b–(1)–(a). Changing containment levels for types of experiments that are specified in the Guidelines when a major action is involved;

IV–C–1–b–(1)–(b). Assigning containment levels for types of experiments that are not explicitly considered in the Guidelines when a major action is involved;

IV–C–1–b–(1)–(c). Promulgating and amending a list of classes of recombinant DNA molecules to be exempt from these Guidelines because they consist entirely of DNA segments from species that exchange DNA by known physiological processes or otherwise do not present a significant risk to health or the environment;

IV–C–1–b–(1)–(d). Permitting experiments specified by Section III–A of the Guidelines;

IV–C–1–b–(1)–(e). Certifying new host-vector systems with the exception of minor modifications of already certified systems (the standards and procedures for certification are described in Appendix I–II–A. Minor modifications constitute, for example, those of minimal or no consequence to the properties relevant to containment); and

IV–C–1–b–(1)–(f). Adopting other changes in the Guidelines.

IV–C–1–b–(2). Lesser Actions. To execute lesser actions, the director must seek the advice of the RAC. The director's decision will be transmitted to the RAC and IBC chairpersons and publiched in the *Recombinant DNA Technical Bulletin:*

IV–C–1–b–(2)–(a). Interpreting and determining containment levels upon request by ORDA;

IV–C–1–b–(2)–(b). Changing containment levels for experiments that are specified in the Guidelines (see Section III);

IV–C–1–b–(2)–(c). Assigning containment levels for experiments not explicitly considered in the Guidelines;

IV–C–1–b–(2)–(d). Revising the "Classification of Etiologic Agents" for the purpose of these Guidelines [1].

IV–C–1–b–(3). Other Actions. The director's decision will be transmittede to the RAC and IBC chairpersons and published in the *Recombinant DNA Technical Bulletin:*

IV–C–1–b–(3)–(a). Interpreting the Guidelines for experiments to which the Guidelines specifically assign containment levels;

IV–C–1–b–(3)–(b). Setting containment under Section III–B–1–d and Section III–B–3–d;

IV–C–1–b–(3)–(c). Approving minor modifications of already certified host-vector systems (the standards and procedures for such modifications are described in Appendix I–II);

IV–C–1–b–(3)–(d). Decertifying already certified host-vector systems;

IV–C–1–b–(3)–(e). Adding new entries to the list of molecules toxic for vertebrates (see Appendix F);

IV–C–1–b–(3)–(f). Approving the cloning of toxin genes in host-vector systems other than *E. coli* K–12 (see Appendix F); and

IV–C–1–b–(3)–(g). Determining appropriate containment conditions for experiments according to case precedents developed under Section IV–C–1–b–(2)–(c).

IV–C–1–b–(4). The director shall conduct, support, and assist training programs in laboratory safety for IBC members, BSOs, PIs, and laboratory staff.

IV–C–2. Recombinant DNA Advisory Committee. The Recombinant DNA Advisory Committee (RAC) is responsible for carrying out specified functions cited below as well as others assigned under its charter or by the Secretary, HHS, the Assistant Secretary for Health, and the Director, NIH.

The committee shall consist of 25 members including the chair, appointed by the Secretary or his or her designee, at least fourteen of whom shall be selected from authorities knowledgeable in the fields of molecular biology or recombinant DNA research or in scientific fields other than molecular biology or recombinant DNA research, and at least six of whom shall be persons knowledgeable in applicable law, standards of professional conduct and practice, public attitudes, the environment, public health, occupational health, or related fields. Representatives from Federal agencies shall serve as non-voting members. Nominations for the RAC may be submitted to the Office of Recombinant DNA Activities, National Institutes of Health, Building 31, Room 3B10, Bethesda, MD 20892.

All meetings of the RAC will be announced in the **Federal Register**, including tentative agenda items, 30 days in advance of the meeting with final agendas (if modified) available at least 72 hours before the meeting. No item defined as a major action under Section IV–C–1–b–(1) may be added to an agenda after it appears in the **Federal Register**.

The RAC shall be responsible for advising the Director, NIH, on the actions listed in Section IV–C–1–b–(1) and IV–C–1–b–(2).

IV–C–3. The Office of Recombinant DNA Activities. The ORDA shall serve as a focal point for information on recombinant DNA activities and provide advice to all within and outside NIH including Institutions, BSOs, PIs, Federal agencies, State and local governments and institutions in the private sector. The ORDA shall carry out such other functions as may be delegated to it by the Director, NIH, including those authorities described in Section IV–C–1–b–(3). In addition, ORDA shall be responsible for the following:

IV–C–3–a. Reviewing and approving IBC membership;

IV–C–3–b. Publishing in the **Federal Register:**

IV–C–3–b–(1). Announcements of RAC meetings and agendas at least 30 days in advance;

Note.—If the agenda for an RAC meeting is modified, ORDA shall make the revised agenda available to anyone upon request at least 72 hours in advance of the meeting.

IV–C–3–b–(2). Proposed major actions of the type falling under Section IV–C–1–b–(1) at least 30 days prior to the RAC meeting at which they will be considered; and

IV–C–3–b–(3). The NIH director's final decision on recommendations made by the RAC.

IV–C–3–c. Publishing the *Recombinant DNA Technical Bulletin;* and

IV–C–3–d. Serving as executive secretary of the RAC.

IV–C–4. Other NIH Components. Other NIH components shall be responsible for certifying maximum containment (BL4) facilities, inspecting them periodically, and inspecting other recombinant DNA facilities as deemed necessary.

IV–D—Compliance

As a condition for NIH funding of recombinant DNA research, institutions must ensure that such research conducted at or sponsored by the institution, irrespective of the source of funding, shall comply with these Guidelines. The policies on noncompliance are as follows:

IV–D–1. All NIH-funded projects involving recombinant DNA techniques must comply with the NIH Guidelines. Noncompliance may result in (i) suspension, limitation, or termination of financial assistance for such projects and of NIH funds for other recombinant DNA research at the institution, or (ii) a requirement for prior NIH approval of any or all recombinant DNA projects at the Institution.

IV–D–2. All non-NIH funded projects involving recombinant DNA techniques conducted at or sponsored by an institution that receives NIH funds for projects involving such techniques must comply with the NIH Guidelines. Noncompliance may result in: (i) Suspension, limitation, or termination of NIH funds for recombinant DNA research at the institution, or (ii) a requirement for prior NIH approval of any or all recombinant DNA projects at the institution.

IV–D–3. Information concerning noncompliance with the Guidelines may be brought forward by any person. It should be delivered to both NIH (ORDA) and the relevant Institution. The institution, generally through the IBC, shall take appropriate action. The institution shall forward a complete report of the incident to ORDA, recommending any further action.

IV–D–4. In cases where NIH proposes to suspend, limit, or terminate financial assistance because of noncompliance with the Guidelines, applicable DHHS and Public Health Service procedures shall govern.

IV–D–5. Voluntary Compliance. Any individual, corporation, or institution that is not otherwise covered by the Guidelines is encouraged to conduct recombinant DNA research activities in accordance with the Guidelines through the procedures set forth in Part VI.

V. Footnotes and References of Sections I–IV

1. The original reference to organisms as Class 1, 2, 3, 4, or 5 refers to the classification in the publication *Classification of Etiologic Agents on the Basis of Hazard,* 4th Edition, July 1974; U.S. Department of Health, Education, and Welfare, Public Health Service, Centers for Disease Control, Office of Biosafety, Atlanta, Georgia 30333.

The Director, NIH, with advice of the Recombinant DNA Advisory Committee, may revise the classification for the purposes of these Guidelines (see Section IV–C–1–b–(2)–(d)). The revised list of organisms in each class is reprinted in Appendix B to these Guidelines.

2. In Part III of the Guidelines, there are a number of places where judgments are to be made. In all these cases the principal investigator is to make the judgment on these matters as part of his responsibility to "make the initial determination of the required levels of physical and biological containment in accordance with the Guidelines" (Section IV–B–5–c–(1)). In the cases falling under Sections III–A, –B or –C, this judgment is to be reviewed and approved by the IBC as part of its responsibility to make "an independent assessment of the containment levels required by these Guidelines for the proposed research" (Section IV–B–3–a–(1)). If the IBC wishes, any specific cases may be referred to ORDA as part of ORDA's functions to "provide advice to all within and outside NIH" (Section IV–C–3), and ORDA may request advice from the RAC as part of the RAC's responsibility for "interpreting and determining containment levels upon request by ORDA" (Section IV–C–1–b–(2)–(a)).

3. *Laboratory Safety at the Center for Disease Control* (Sept. 1974). U.S. Department of Health, Education and Welfare Publication No. CDC 75–8118.

4. *Classification of Etiologic Agents on the Basis of Hazard* (4th Edition, July 1974). U.S. Department of Health, Education and Welfare. Public Health Service. Centers for Disease Control, Office of Biosafety, Atlanta, Georgia 30333.

5. *National Cancer Institute Safety Standards for Research Involving Oncogenic Viruses* (Oct. 1974). U.S. Department of Health, Education and Welfare Publication No. (NIH) 75–790.

6. *National Institutes of Health Biohazards Safety Guide* (1974). U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health. U.S. Government Printing Office, Stock No. 1740–00383.

7. *Biohazards in Biological Research* (1973). A. Hellman, M.N. Oxman, and R. Pollack (ed.) Cold Spring Harbor Laboratory.

8. *Handbook of Laboratory Safety* (1971). 2nd Edition. N.V. Steere (ed.). The Chemical Rubber Co., Cleveland.

9. Bodily, J.L. (1970). *General Administration of the Laboratory,* H.L. Bodily, E.L. Updyke, and J.O. Mason (eds.), Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections. American Public Health Association, New York, pp. 11–28.

10. Darlow, H.M. (1969). *Safety in the Microbiological Laboratory.* In J.R. Norris and D.W. Robbins (ed.), Methods in Microbiology. Academic Press, Inc., New York, pp. 169–204.

11. *The Prevention of Laboratory Acquired Infection* (1974). C.H. Collins, E.G. Hartley, and R. Pilsworth. Public Health Laboratory Service, Monograph Series No. 6.

12. Chatigny, M.A. (1961). *Protection Against Infection in the Microbiological Laboratory: Devices and Procedures.* In W.W. Umbreit (ed.): Advances in Applied Microbiology. Academic Press, New York, N.Y. 3:131–192.

13. *Design Criteria for Viral Oncology Research Facilities* (1975). U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, DHEW Publication No. (NIH) 75–891.

14. Kuehne, R.W. (1973). *Biological Containment Facility for Studying Infectious Disease.* Appl. Microbiol. 28–239–243.

15. Runkle, R.S., and G.B. Phillips (1969). *Microbial Containment Control Facilities.* Van Nostrand Reinhold, New York.

16. Chatigny, M.A., and D.L Clinger (1969). *Contamination Control in Aerobiology.* In R.L. Dimmick and A.B. Akers (eds.). An Introduction to Experimental Aerobiology. John Wiley & Sons, New York, pp. 194–263.

17. As classified in the Third Report of the International Committee on Taxonomy of Viruses: Classification and Nomenclature of Viruses, R.E.F. Matthews, Ed. Intervirology 12 (129–296) 1979.

18. A USDA permit, required for import and interstate transport of pathogens, may be obtained from the Animal and Plant Health Inspection Service, USDA, Federal Building, Hyattsville, MD 20782.

19. i.e., the total of all genomes within a Family shall not exceed two-thirds of the genome.

20. All activities, including storage of variola and whitepox, are restricted to the single national facility (World Health Organization (WHO) Collaborating Center for Smallpox Research, Centers for Disease Control, in Atlanta).

21. Section III–A–4 covers only those experiments in which the intent is to modify stably the genome of cells of a human subject. Other experiments involving recombinant DNA in human subjects such as feeding of bacteria containing recombinant DNA or the administration of vaccines containing recombinant DNA are not covered in Section III–A–4 of the Guidelines.

22. For recombinant DNA experiments in which the intent is to modify stably the genome of cells of a human subject, see Section III–A–4.

VI. Voluntary Compliance

VI-A.—Basic Policy

Individuals, corporations, and institutions not otherwise covered by the Guidelines are encouraged to do so by following the standards and procedures set forth in Parts I–IV of the Guidelines. In order to simplify discussion, references hereafter to "institutions" are intended to encompass corporations, and individuals who have no organizational affiliation. For purposes of complying with the Guidelines, an individual intending to carry out research involving recombinant DNA is encouraged to affiliate with an institution that has an IBC approved under the Guidelines.

Since commercial organizations have special concerns, such as protection of proprietary data, some modifications and explanations of the procedures in Parts I–IV are provided below, in order to address these concerns.

VI-B—IBC Approval

The ORDA will review the membership of an institution's IBC, and where it finds the IBC meets the requirements set forth in Section IV–B–2 will give its approval to the IBC membership.

It should be emphasized that employment of an IBC member solely for purposes of membership on the IBC does not itself make the member an institutionally affiliated member for purposes of Section IV–B–2–a.

Except for the unaffiliated members, a member of an IBC for an institution not otherwise covered by the Guidelines may participate in the review and approval of a project in which the member has a direct financial interest so long as the member has not been, and does not expect to be, engaged in the project. Section IV–B–2–d is modified to that extent for purposes of these institutions.

VI-C—Certification of Host-Vector Systems

A host-vector system may be proposed for certification by the Director, NIH, in accordance with the procedures set forth in Appendix I–II–A.

In order to ensure protection for proprietary data, any public notice regarding a host-vector system which is designated by the institution as proprietary under Section VI–E–1 will be issued only after consultation with the institution as to the content of the notice.

VI-D—Requests for Exemptions and Approvals

Requests for exemptions or other approvals required by the Guidelines should be requested by following the procedures set forth in the appropriate sections in Parts I–IV of the Guidelines.

In order to ensure protection for proprietary data, any public notice regarding a request for an exemption or other approval which is designated by the institution as proprietary under Section VI–E–1 will be issued only after consultation with the institution as to the content of the notice.

VI-E—Protection of Proprietary Data

In general, the Freedom of Information Act requires Federal agencies to make their records available to the public upon request. However, this requirement does not apply to, among other things, "trade secrets and commercial and financial information obtained from a person and privileged or confidential." 18 U.S.C. 1905, in turn makes it a crime for an officer or employee of the United States or any Federal department or agency to publish, divulge, disclose, or make known "in any manner or to any extent not authorized by law any information coming to him in the course of his employment or official duties or by reason of any examination or investigation made by, or return, report or record made to or filed with, such department or agency or officer or employee thereof, which information concerns or relates to the trade secrets, [or] processes . . . of any person, firm, partnership, corporation, or association." This provision applies to all employees of the Federal Government, including special Government employees. Members of the Recombinant DNA Advisory Committee are "special Government employees."

VI-E-1. In submitting to NIH for purposes of complying voluntarily with the Guidelines, an institution may designate those items of information which the institution believes constitute trade secrets, privileged, confidential commercial, or financial information.

VI-E-2. If NIH receives a request under the Freedom of Information Act for information so designated, NIH will promptly contact the institution to secure its views as to whether the information (or some portion) should be released.

VI-E-3. If the NIH decides to release this information (or some portion) in response to a Freedom of Information request or otherwise, the institution will be advised; and the actual release will not be made until the expiration of 15 days after the institution is so advised except to the extent that earlier release in the judgment of the Director, NIH, is necessary to protect against an imminent hazard to the public or the environment.

VI-E-4. Presubmission Review.

VI-E-4-a. Any institution not otherwise covered by the Guidelines, which is considering submission of data or information voluntarily to NIH, may request presubmission review of the records involved to determine whether if the records are submitted NIH will or will not make part or all of the records available upon request under the Freedom of Information Act.

VI-E-4-b. A request for presubmission review should be submitted to ORDA along with the records involved. These records must be clearly marked as being the property of the institution on loan to NIH solely for the purpose of making a determination under the Freedom of Information Act. The ORDA will then seek a determination from the HHS Freedom of Information Officer, the responsible official under HHS regulations (45 CFR Part 5) as to whether the records involved (or some portion) are or are not available to members of the Public under the Freedom of Information Act. Pending such a determination the records will be kept separate from ORDA files, will be considered records of the institution and not ORDA, and will not be received as part of ORDA files. No copies will be made of the records.

VI-E-4-c. The ORDA will inform the institution of the HHS Freedom of Information Officer's determination and follow the institution's instructions as to whether some or all of the records involved are to be returned to the institution or to become a part of ORDA files. If the institution instructs ORDA to return the records, no copies or summaries of the records will be made or retained by HHS, NIH, or ORDA.

VI-E-4-d. The HHS Freedom of Information Officer's determination will represent that official's judgement at the time of the determination as to whether the records involved (or some portion) would be exempt from disclosure under the Freedom of Information Act if at the time of the determination the records were in ORDA files at a request were received for them under the Act.

Appendix A—Exemptions Under Section III–D–4

Section III–D–4 states that exempt from these Guidelines are "certain specified recombinant DNA molecules that consist entirely of DNA segments from different species that exchange

DNA by known physiological processes though one or more of the segments may be a synthetic equivalent. A list of such exchangers will be prepared and periodically revised by the Director, NIH, with advice of the RAC after appropriate notice and opportunity for public comment (see Section IV–C–1–b–(1)–(c)). Certain classes are exempt as of publication of these revised Guidelines. The list is in Appendix A."

Under Section III–D–4 of these Guidelines are recombinant DNA molecules that are: (1) Composed entirely of DNA segments from one or more of the organisms within a sublist and (2) to be propagated in any of the organisms within a sublist. (Classification of *Bergey's Manual of Determinative Bacteriology*, 8th edition. R. E. Buchanan and N. E. Gibbons, editors. Williams and Wilkins Company: Baltimore, 1974.)

Although these experiments are exempt, it is recommended that they be performed at the appropriate biosafety level for the host or recombinant organism (for biosafety levels see *Biosafety in Microbiological and Biomedical Laboratories*, 1st Edition (March 1984), U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Georgia 30333, and National Institutes of Health, Bethesda, Maryland 20892).

Sublist A

1. Genus *Escherichia*
2. Genus *Shigella*
3. Genus *Salmonella* (including *Arizona*)
4. Genus *Enterobacter*
5. Genus *Citrobacter* (including *Levinea*)
6. Genus *Klebsiella*
7. Genus *Erwinia*
8. *Pseudomonas aeruginosa, Pseudomonas Putida* and *Pseudomonas fluorescens*
9. *Serratia marcescens*
10. *Yersinia enterocolitica*

Sublist B

1. *Bacillus subtilis*
2. *Bacillus licheniformis*
3. *Bacillus pumilus*
4. *Bacillus globigii*
5. *Bacillus niger*
6. *Bacillus natо*
7. *Bacillus amyloliquefaciens*
8. *Bacillus atterrimus*

Sublist C

1. *Streptomyces aureofaciens*
2. *Streptomyces rimosus*
3. *Streptomyces coelicolor*

Sublist D

1. *Streptomyces griseus*
2. *Streptomyces cyaneus*
3. *Streptomyces venezuelae*

Sublist E

1. One way transfer of *Streptococcus mutans* or *Streptococcus lactis* DNA into *Streptococcus sanguis*.

Sublist F

1. *Streptococcus sanguis*
2. *Streptococcus pneumoniae*
3. *Streptococcus faecalis*
4. *Streptococcus pyogenes*
5. *Streptococcus mutans*

APPENDIX B—CLASIFICATION OF MICROORGANISMS ON THE BASIS OF HAZARD

Appendix B–I—Classification of Etiologic Agents

The original reference for this classification was the publication *Classification of Etiological Agents on the Basis of Hazard*, 4th edition, July 1974, U.S. Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, Office of Biosafety; Atlanta, Georgia 30333. For the purposes of these Guidelines, this list has been revised by the NIH [1].

Appendix B–I–A. Class 1 Agents. All bacterial, parasitic, fungal, viral, rickettsial, and chlamydial agents not included in higher classes.

Appendix B–I–B. Class 2 Agents.

Appendix B–I–B–1. Bacterial Agents.

Acinetobacter calcoaceticus
Actinobacillus-all species
Aeromonas hydrophila
Arizona hinshawii-all serotypes
Bacillus anthracis
Bordetella-all species
Borrelia recurrentis, B. vincenti
Campylobacter fetus
Campylobacter jejuni
Chlamydia psittaci
Chlamydia trachomatis
Clostridium botulinum, Cl. chauvoei, Cl. haemolyticum, Cl. histolyticum, Cl. novyi, Cl. septicum, Cl. tetani
Corynebacterium diphtheriae, C. equi, C. haemolyticum, C. pseudotuberculosis, C. pyogenes, C. renale
Edwardsiella tarda
Erysipelothrix insidiosa
Escherichia coli-all enteropathogenic, enterotoxigenic, enteroinvasive and strains bearing K1 antigen
Haemophilus ducreyi, H. influenzae
Klebsiella-all species and all serotypes
Legionella pneumophila
Leptospira interrogans-all serotypes
Listeria-all species
Moraxella-all species
Mycobacteria-all species except those listed in Class 3
Mycoplasma-all species except *Mycoplasma mycoides* and *Mycoplasma agalactiae*, which are in Class 5
Neisseria gonorrhoeae, N. meningitidis
Pasteurella-all species except those listed in Class 3
Salmonella-all species and all serotypes
Shigella-all species and all serotypes
Sphaerophorus necrophorus
Staphylococcus aureus
Streptobacillus moniliformis
Streptococcus pneumoniae
Streptococcus pyogenes
Treponema carateum, T. pallidum, and T. pertenue
Vibrio cholerae
Vibrio parahemolyticus
Yersinia enterocolitica

Appendix B–I–B–2. Fungal Agents.

Actinomycetes (including *Nocardia* species, *Actinomyces* species, and *Arachnia propionica*) [2]
Blastomyces dermotitidis
Cryptococcus neoformans
Paracoccidioides braziliensis

Appendix B–I–B–3. Parasitic Agents.

Endamoeba histolytica
Leishmonia sp.
Naegleria gruberi
Schistosoma monsoni
Toxoplasma gondii
Toxocara canis
Trichinella spiralis
Trypanosoma cruzi

Appendix B–I–B–4. Viral, Rickettsial, and Chlamydial Agents.

Adenoviruses—human—all types
Cache Valley virus
Coxsackie A and B viruses
Cytomegoloviruses
Echoviruses—all types
Encephalomyocarditis virus (EMC)
Flanders virus
Hart Pork virus
Hepotitus—associated antigen material
Herpes viruses-except *Herpesvirus simiae (Monkey B virus)* which is in Class 4
Corona viruses
Influenza viruses—all types except A/PR8/34, which is in Class 1
Langot virus
Lymphogranuloma venereum agent
Measles virus
Mumps virus
Parainfluenza virus—all types except Parainfluenza virus 3, SF4 strain, which is in Class 1
Polioviruses—all types, wild and attenuated
Paxviruses—all types except *Alastrim, Smallpox*, and *Whitepox* which are Class 5 and *Monkey pox* which depending on experiments is in Class 3 or Class 4
Rabies virus—all strains except *Rabies street virus* which should be classified in Class 3
Reoviruses—all types
Respiratory syncytiol virus
Rhinoviruses—all types
Rubella virus
Simian viruses—all types except *Herpesvirus simiae (Monkey B virus)* and *Marburg virus* which are in Class 4
Sindbis virus
Tensaw virus
Turlock virus
Vaccinia virus
Varicella virus
Vesicular stomotitis virus [3]

Vole rickettsia
Yellow fever virus, 17D vaccine strain

Appendix B–I–C. Class 3 Agents.

Appendix B–I–C–1. Bacterial Agents.

Bartonella—all species
Brucella—all species
Francisella tularensis
Mycobacterium avium, M. bovis, M. tuberculosis
Pasteurella multocide type *B* ("buffalo" and other foreign virulent strains) [3]
Pseudomonas mallei [3]
Pseudomonas pseudomallei [3]
Yersinia pestis

Appendix B–I–C–2. Fungal Agents.

Coccidioides immitis
Histoplasma capsulatum
Histoplasma capsulatum var. *duboisii*

Appendix B–I–C–3. Parasitic Agents.

None.

Appendix B–I–C–4. Viral, Rickettsial, and Chlamydial Agents.

Monkey pox, when used *in vitro* [4]
Arboviruses-all strains except those in Class 2 and 4 (*Arboviruses* indigenous to the United States are in Class 3 except those listed in Class 2. *West Nile* and *Semliki Forest* viruses may be classified up or down depending on the conditions of use and geographical location of the laboratory.)
Dengue virus, when used for transmission or animal inoculation experiments
Lymphocytic choriomeningitis virus (LCM)
Rickettsia—all species except *Vole rickettsia* when used for transmission or animal inoculation experiments
Yellow fever virus—wild, when used *in vitro*

Appendix B–I–D. Class 4 Agents.

Appendix B–I–D–1. Bacterial Agents.

None.

Appendix B–I–D–2. Fungal Agents.

None.

Appendix B–I–D–3. Parasitic Agents.

None.

Appendix B–I–D–4. Viral, Rickettsial, and Chlamydial Agents.

Ebola fever virus
Monkey pox, when used for transmission or animal inoculation experiments [4]
Hemorrhagic fever agents, including *Crimean hemorrhagic fever, (Congo), Junin*, and *Machupo* viruses, and others as yet undefined
Herpesvirus simiae (Monkey B virus)
Lassa virus
Marburg virus
Tick-borne encephalitis virus complex, including *Russian spring-summer encephalitis, Kyasanur forest disease, Omsk hemorrhagic fever*, and *Central European encephalitis viruses*
Venezuelan equine encephalitis virus, epidemic strains, when used for transmission or animal inoculation experiments
Yellow fever virus—wild, when used for transmission or animal inoculation experiments

Appendix B–II—Classification of Oncogenic Viruses on the Basis of Potential Hazard [5]

Appendix B–II–A. Low-Risk Oncogenic Viruses.

Rous sarcoma
SV–40
CELO
Ad7–SV40
Polyoma
Bovine papilloma
Rat mammary tumor
Avian leukosis
Murine leukemia
Murine sarcoma
Mouse mammary tumor
Rat leukemia
Hamster leukemia
Bovine leukemia
Dog sarcoma
Mason-Pfizer monkey virus
Marek's
Guinea pig herpes
Lucke (Frog)
Adenovirus
Shope fibroma
Shope papilloma

Appendix B–II–B. Moderate-Risk Oncogenic Viruses.

Ad2–SV40
FeLV
HV Saimiri
EBV
SSV–1
GaLV
HV ateles
Yaba
FeSV

Appendix B–III—Class 5 Agents

Appendix B–III–A. Animal Disease Organisms Which are Forbidden Entry into the United States by Law.

Foot and mouth disease virus.

Appendix B–III–B. Animal Disease Organisms and Vectors Which are Forbidden Entry into the United States by USDA Policy.

African horse sickness virus
African swine fever virus
Besnoitia besnoiti
Borna disease virus
Bovine infectious petechial fever
Camel pox virus
Ephemeral fever virus
Fowl plague virus
Goat pox virus
Hog cholera virus
Louping ill virus
Lumpy skin disease virus
Nairobi sheep disease virus
Newcastle disease virus (Asiatic strains)
Mycoplasma mycoides (contagious bovine pleuropneumonia)
Mycoplasma agalactiae (contagious agalactia of sheep)
Rickettsia ruminatium (heart water)
Rift valley fever virus
Rhinderpest virus
Sheep pox virus
Swine vesicular disease virus
Teschen disease virus
Trypanosoma vivax (Nagana)
Trypanosoma evansi
Theileria parva (East Coast fever)
Theileria annulata
Theileria lawrencei
Theileria bovis
Theileria hirci
Vesicular exanthema virus
Wesselsbron disease virus
Zyonema

Appendix B–III–C. Organisms Which may not be Studied in the United States Except at Specified Facilities.

Small pox [4]
Alastrim [4]
White pox [4]

Appendix B–IV—Footnotes and References of Appendix B.

1. The original reference for this classification was the publication *Classification of Etiologic Agents on the Basis of Hazard*, 4th edition, July 1974, U.S. Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, Office of Biosafety, Atlanta, Georgia 30333. For the purposes of these Guidelines, this list has been revised by the NIH.

2. Since the publication of the classification in 1974 [1], the *Actinomycetes* have been reclassified as bacterial rather than fungal agents.

3. A USDA permit, required for import and interstate transport of pathogens, may be obtained from the Animal and Plant Health Inspection Service, USDA, Federal Building, Hyattsville, MD 20782.

4. All activities, including storage of variola and whitepox, are restricted to the single national facility [World Health Organization (WHO) Collaborating Center for Smallpox Research, Centers for Disease Control, in Atlanta].

5. *National Cancer Institute Safety Standards for Research Involving Oncogenic Viruses* (October 1974). U.S. Department of Health, Education, and Welfare Publication No. (NIH) 75–790.

6. U.S. Department of Agriculture, Animal and Plant Health Inspection Service.

Appendix C—Exemptions Under Section III–D–5

Section III–D–5 states that exempt from these Guidelines are "Other classes of recombinant DNA molecules if the Director, NIH, with advice of the RAC, after appropriate notice and opportunity for public comment finds that they do not present a significant risk to health or the environment (see Section IV–C–1–b–(1)–(c)). Certain classes are exempt as of publication of these revised Guidelines."

The following classes of experiments are exempt under Section III–D–5 of the Guidelines:

Appendix C–I—Recombinant DNAs in Tissue Culture.

Recombinant DNA molecules containing less than one-half of any eukaryotic genome (all viruses from a single Family (4) being considered identical (5)) that are propagated and maintained in cells in tissue culture are exempt from these Guidelines with the exceptions listed below.

Exceptions. Experiments described in Section III–A which require specific RAC review and NIH approval before initiation of the experiment.

Experiments involving DNA from Class 3, 4, or 5 organisms [1] or cells known to be infected with these agents.

Experiments involving the deliberate introduction of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F).

Appendix C–II—Experiments Involving E. coli K–12 Host-Vector Systems

Experiments which use *E. coli* K–12 host-vector systems, with the exception of those experiments listed below, are exempt from these Guidelines provided that: (i) the *E. coli* host shall not contain conjugation proficient plasmids or generalized transducing phages; and (ii) lambda or lambdoid or Ff bacteriophages or nonconjugative plasmids [2] shall be used as vectors. However, experiments involving the insertion into *E. coli* K–12 of DNA from prokaryotes that exchange genetic information [3] with *E. coli* may be performed with any *E. coli* K–12 vector (e.g., conjugative plasmid). When a nonconjugative vector is used, the *E. coli* K–12 host may contain conjugation-proficient plasmids either autonomous or integrated, or generalized transducing phages.

For these exempt laboratory experiments, BL1 physical containment conditions are recommended.

For large-scale (LS) fermentation experiments BL1–LS physical containment conditions are recommended. However, following review by the IBC of appropriate data for a particular host-vector system, some latitude in the application of BL1–LS requirements as outlined in Appendix K–II–A through K–II–F is permitted.

Exceptions. Experiments described in Section III–A which require specific RAC review and NIH approval before initiation of the experiment.

Experiments involving DNA from Class 3, 4, or 5 organisms [1] or from cells known to be infected with these agents may be conducted under containment conditions specified in Section III–B–2 with prior IBC review and approval.

Large-scale experiments (e.g., more than 10 liters of culture) require prior IBC review and approval (see Section III–B–5).

Experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F).

Appendix C–III—Experiments Involving Saccharomyces Host-Vector Systems

Experiments which use *Saccharomyces cerevisiae* host-vector systems, with the exception of experiments listed below, are exempt from these Guidelines.

Experiments which use *Saccharomyces uvarum* host-vector systems, with the exception of experiments listed below, are exempt from these Guidelines.

For these exempt laboratory experiments, BL1 physical containment conditions are recommended.

For large-scale fermentation experiments BL1–LS physical containment conditions are recommended. However, following review by the IBC of appropriate data for a particular host-vector system some latitude in the application of BL1–LS requirements as outlined in Appendix K–II–A through K–II–F is permitted.

Exceptions. Experiments described in Section III–A which require specific RAC review and NIH approval before initiation of the experiment.

Experiments involving Class 3, 4, or 5 organisms [1] or cells knowns to be infected with these agents may be conducted under containment conditions specified in Section III–B–2 with prior IBC review and approval.

Large-scale experiments (e.g., more than 10 liters of culture) require prior IBC review and approval (see Section III–B–5).

Experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F).

Appendix C–IV—Experiments Involving Bacillus subtilis Host-Vector Systems

Any asporogenic *Bacillus subtilis* strain which does not revert to a sporeformer with a frequency greater than 10^{-7} can be used for cloning DNA with the exception of those experiments listed below.

For these exempt laboratory experiments, BL1 physical containment conditions are recommended.

For large-scale fermentation experiments BL1–LS physical containment conditions are recommended. However, following review by the IBC of appropriate data for a particular host-vector system, some latitude in the application of BL1–LS requirements as outlined in Appendix K–II–A through K–II–F is permitted.

Exceptions. Experiments described in Section III–A which require specific RAC review and approval before initiation of the experiment.

Experiments involving Class 3, 4, or 5 organisms [1] or cells known to be infected with these agents may be conducted under containment conditions specified by Section III–B–2 with prior IBC review and approval.

Large-scale experiments (e.g., more than 10 liters of culture) require prior IBC review and approval (see Section III–B–5).

Experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F).

Appendix C–V—Extrachromosomal Elements of Gram Positive Organisms

Recombinant DNA molecules derived entirely from extrachromosomal elements of the organisms listed below (including shuttle vectors constructed from vectors described in Appendix C), propagated and maintained in organisms listed below are exempt from these Guidelines.

Bacillus subtilis
Bacillus pumilus
Bacillus licheniformis
Bacillus thuringiensis
Bacillus cereus
Bacillus amyloliquefaciens
Bacillus brevis
Bacillus natto
Bacillus niger
Bacillus aterrimus
Bacillus amylosaccharitícus
Bacillus anthracis
Bacillus globigii
Bacillus megaterium
Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus carnosus
Clostridium acetobutylicum
Pediococcus damnosus
Pediococcus pentosaceus
Pediococcus acidilactici
Lactobacillus casei
Listeria grayi
Listeria murrayi
Listeria monocytogenes
Streptococcus pyogenes
Streptococcus agalactiae
Streptococcus sanguis
Streptococcus salivarious
Streptococcus cremoris
Streptococcus pneumoniae
Streptococcus avium
Streptococcus faecalis
Streptococcus anginosus
Streptococcus sobrinus
Streptococcus lactis
Streptococcus mutans
Streptococcus equisimilis
Streptococcus thermophylus

Streptococcus milleri
Streptococcus durans
Streptococcus mitior
Streptococcus ferus

Exceptions. Experiments described in Section III–A which require specific RAC review and NIH approval before initiation of the experiment.

Large-scale experiments (e.g., more than 10 liters of culture) require prior IBC review and approval (see Section III–B–5).

Experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates (see Appendix F).

Appendix C–VI—Footnotes and References of Appendix C

1. The original reference to organisms as Class 1, 2, 3, 4, or 5 refers to the classification in the publication *Classification of Etiologic Agents on the Basis of Hazard*, 4th Edition, July 1974; U.S. Department of Health, Education and Welfare, Public Health Service, Centers for Disease Control, Office of Biosafety, Atlanta, Georgia 30333.

The Director, NIH, with advice of the Recombinant DNA Advisory Committee, may revise the classification for the purposes of these Guidelines (see Section IV–C–1–b–(2)–(d)). The revised list of organisms in each class is reprinted in Appendix B to these Guidelines.

2. A subset of non-conjugative plasmid vectors are also poorly mobilizable (e.g., pBR322, pBR313). Where practical, these vectors should be employed.

3. Defined as observable under optimal laboratory conditions by transformation, transduction, phage infection, and/or conjugation with transfer of phage, plasmid, and/or chromosomal genetic information. Note that this definition of exchange may be less stringent than that applied to exempt organisms under Section III–D–4.

4. As classified in the Third Report of the International Committee on Taxonomy of Viruses: Classification and Nomenclature of Viruses, R.E.F. Matthews, Ed. Intervirology 12 (129–296) 1979.

5. i.e., the total of all genomes within a Family shall not exceed one-half of the genome.

Appendix D—Actions Taken Under the Guidelines

As noted in the subsections of Section IV–C–1–b–(1), the Director, NIH, may take certain actions with regard to the Guidelines after the issues have been considered by the RAC. Some of the actions taken to date include the following:

Appendix D–I

Permission is granted to clone foot and mouth disease virus in the EK1 host-vector system consisting of *E. coli* K–12 and the vector pBR322, all work to be done at the Plum Island Animal Disease Center.

Appendix D–II

Certain specified clones derived from segments of the foot and mouth disease virus may be transferred from Plum Island Animal Disease Center to the facilities of Genentech, Inc., of South San Francisco, California. Further development of the clones at Genentech has been approved under BL1+EK1 conditions.

Appendix D–III

The Rd strain of *Hemophilus influenzae* can be used as a host for the propagation of the cloned Tn 10 tet R gene derived from *E. coli* K–12 employing the non-conjugative *Hemophilus* plasmid, pRSF0885, under BL1 conditions.

Appendix D–IV

Permission is granted to clone certain subgenomic segments of foot and mouth disease virus in HV1 *Bacillus subtilis* and *Saccharomyces cerevisiae* host-vector systems under BL1 conditions at Genentech, Inc., South San Francisco, California.

Appendix D–V

Permission is granted to Dr. Ronald Davis of Stanford University to field test corn plants modified by recombinant DNA techniques under specified containment conditions.

Appendix D–VI

Permission is granted to clone in *E. coli* K–12 under BL1 physical containment conditions subgenomic segments of rift valley fever virus subject to conditions which have been set forth by the RAC.

Appendix D–VII

Attenuated laboratory strains of *Salmonella typhimurium* may be used under BL1 physical containment conditions to screen for the *Saccharomyces cerevisiae* pseudouridine synthetase gene. The plasmid YEp13 will be employed as the vector.

Appendix D–VIII

Permission is granted to transfer certain clones of subgenomic segments of foot and mouth disease virus from Plum Island Animal Disease Center to the laboratories of Molecular Genetics, Inc., Minnetonka, Minnesota, and to work with these clones under BL1 containment conditions. Approval is contingent upon review of data on infectivity testing of the clones by a working group of the RAC.

Appendix D–IX

Permission is granted to Dr. John Sanford of Cornell University to field test tomato and tobacco plants transformed with bacterial (*E. coli* K–12) and yeast DNA using pollen as a vector.

Appendix D–X

Permission is granted to Drs. Steven Lindow and Nickolas Panopoulos of the University of California, Berkeley, to release under specified conditions *Pseudomonas syringae* pv. *syringae* and *Erwinia herbicola* carrying *in vitro* generated deletions of all or part of the genes involved in ice nucleation.

Appendix D–XI

Agracetus of Middleton, Wisconsin, may field test under specified conditions disease resistant tobacco plants prepared by recombinant DNA techniques.

Appendix E—Certified Host-Vector Systems

(See also Appendix I)

While many experiments using *E. coli* K–12, *Saccharomyces cerevisiae* and *Bacillus subtilis* are currently exempt from the Guidelines under Section III–D–5, some derivatives of these host-vector systems were previously classified as HV1 or HV2. A listing of those systems follows:

Appendix E–I—Bacillus subtilis

HV1. The following plasmids are accepted as the vector components of certified *B. subtilis* HV1 systems: pUB110, pC194, pS194, pSA2100, pE194, pT127, pUB112, pC221, pC223, and pAB124. *B. subtilis* strains RUB 331 and BGSC 1S53 have been certified as the host component of HV1 systems based on these plasmids.

HV2. The asporogenic mutant derivative of *Bacillus subtilis*, ASB 298, with the following plasmids as the vector component: pUB110, pC194, pS194, pSA2100, pE194, pT127, pUB112, pC221, pC223, and pAB124.

Appendix E–II—Saccharomyces cerevisiae

HV2. The following sterile strains of *Saccharomyces cerevisiae*, all of which have the ste–VC9 mutation, SHY1, SHY2, SHY3, and SHY4. The following plasmids are certified for use: YIp1, YEp2, YEp4, YIp5, YEp6, YRp7, YEp20, YEp21, YEp24, YIp25, YIp26, YIp27, YIp28, YIp29, YIp30, YIp31, YIp32, and YIp33.

Appendix E–III—Escherichia coli

EK2 Plasmid Systems. The *E. coli* K–12 strain chi–1776. The following

plasmids are certified for use: pSC101, pMB9, pBR313, pBR322, pDH24, pBR325, pBR327, pGL101, and pHB1. The following *E. coli/S. cerevisiae* hybrid plasmids are certified as EK2 vectors when used in *E. coli* chi-1776 or in the sterile yeast strains, SHY1, SHY2, SHY3, and SHY4: YIp1, YEp2, YEp4, YIp5, YEp6, YRp7, YEp20, YEp21, YEp24, YIp25, YIp26, YIp27, YIp28, YIp29, YIp30, YIp31, YIp32, and YIp33.

EK2 Bacteriophage Systems. The following are certified EK2 systems based on bacteriophage lambda:

Vector	*Host*
λgt*WES*·λB′	DP50*supF*
λgt *WES*λB★	DP50*supF*
λgt Zj *vir*λB′	*E. coli* K-12
λgtALO·λB	DP50*supF*
Charon 3A	DP50 or DP50*supF*
Charon 4A	DP50 or DP50*supF*
Charon 16A	DP50 or DP50*supF*
Charon 21A	DP50*supF*
Charon 23A	DP50 or DP50*supF*
Charon 24A	DP50 or DP50*supF*

E. coli K-12 strains chi-2447 and chi-2281 are certified for use with lambda vectors that are certified for use with strain DP50 or DP50*supF* provided that the su⁻ strain not be used as a propagation host.

Appendix E-IV—Neurospora crassa

HV1. The following specified strains of *Neurospora crassa* which have been modified to prevent aerial dispersion:

Inl (inositolless) strains 37102, 37401, 46316, 64001, and 89601.

Csp-1 strain UCLA37 and csp-2 strains FS 590, UCLA101 (these are conidial separation mutants).

Eas strain UCLA191 (an "easily wettable" mutant).

Appendix E-V—Streptomyces

HV1. The following *Streptomyces* species: *Streptomyces coelicolor, S. lividans, S. parvulus,* and *S. griseus.* The following are accepted as vector components of certified *Streptomyces* HV1 systems: *Streptomyces* plasmids SCP2, SLP1.2, pIJ101, actinophage phi C31, and their derivatives.

Appendix E-VI—Pseudomonas putida

HV1. Pseudomonas putida strains KT2440 with plasmid vectors pKT262, pKT263, and pKT264.

Appendix F—Containment Conditions for Cloning of Genes Coding for the Biosynthesis of Molecules Toxic for Vertebrates

Appendix F-I—General Information.

Appendix F specifies the containment to be used for the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates. The cloning of genes coding for molecules toxic for vertebrates that have an LD_{50} of less than 100 nanograms per kilogram body weight (e.g., microbial toxins such as the botulinum toxins, tetanus toxin, diphtheria toxin, *Shigella dysenteriae* neurotoxin) is covered under Section III-A-1 of the Guidelines and requires RAC review and NIH and IBC approval before initiation. No specific restrictions shall apply to the cloning of genes if the protein specified by the gene has an LD_{50} of 100 micrograms or more per kilogram of body weight. Experiments involving genes coding for toxic molecules with an LD_{50} of 100 micrograms or less per kilogram body weight shall be registered with ORDA prior to initiating the experiments. A list of toxic molecules classified as to LD_{50} is available from ORDA. Testing precedures for determining toxicity of toxic molecules not on the list are available from ORDA. The results of such tests shall be forwarded to ORDA which will consult with the RAC Working Group on Toxins prior to inclusion of the molecules on the list (see Section IV-C-1-b-(2)-(e)).

Appendix F-II—Containment Conditions for Cloning of Toxic Molecule Genes in E. coli K-12

Appendix F-II-A. Cloning of genes coding for molecules toxic for vertebrates that have an LD_{50} in the range of 100 nanograms to 1000 nanograms per kilogram body weight (e.g., abrin, *Clostridium perfringens* epsilon toxin) may proceed under BL2+EK2 or BL3+EK1 containment conditions.

Appendix F-II-B. Cloning of genes for the biosynthesis of molecules toxic for vertebrates with an LD_{50} in the range of 1 microgram to 100 micrograms per kilogram body weight may proceed under BL1+EK1 containment conditions (e.g., *Staphylococcus aureus* alpha toxin, *Staphylococcus aureus* beta toxin, ricin, *Pseudomonas aeruginosa* exotoxin A, *Bordatella pertussis* toxin, the lethal factor of *Bacillus anthracis,* the *Pasteurella pestis* murine toxins, the oxygen-labile hemolysins such as streptolysin O, and certain neurotoxins present in snake venoms and other venoms).

Appendix F-II-C. Some enterotoxins are substantially more toxic when administered enterally than parenterally. The following enterotoxins shall be subject to BL1+EK1 containment conditions: cholera toxin, the heat labile toxins of *E. coli, Klebsiella,* and other related proteins that may be identified by neutralization with an antiserum monospecific for cholera toxin, and the heat stable toxins of *E. coli* and of *Yersinia enterocolitica.*

Appendix F-III—Containment Conditions for Cloning of Toxic Molecule Genes in Organisms Other Than E. coli K-12

Requests involving the cloning of genes coding for molecules toxic for vertebrates in host-vector systems other than *E. coli* K-12 will be evaluated by ORDA which will consult with the Working Group on Toxins (see Section IV-C-1-b-(3)-(f)).

Appendix F-IV—Specific Approvals

Appendix F-IV-A. Permission is granted to clone the Exotoxin A gene of *Pseudomonas aeruginosa* under BL1 conditions in *Pseudomonas aeruginosa* and in *Pseudomonas putida.*

Appendix F-IV-B. The pyrogenic exotoxin type A (Tox A) gene of *Staphylococcus aureus* may be cloned in an HV2 *Bacillus subtilis* host-vector system under BL3 containment conditions.

Appendix F-IV-C. Restriction fragments of *Corynephage Beta* carrying the structural gene for diphtheria toxin may be safely cloned in *e. coli* K-12 in high containment Building 550 at the Frederick Cancer Research Facility. Laboratory practices and containment equipment are to be specified by the IBC. If the investigators wish to proceed with the experiments, a prior review will be conducted to advise NIH whether the proposal has sufficient scientific merit to justify the use of the NIH BL4 facility.

Appendix F-IV-D. The genes coding for the *Staphylococcus aureus* determinants, A, B, and F, which may be implicated in toxic shock syndrome may be cloned in *E. coli* K-12 under BL2+EK1 conditions. The *Staphylococcus aureus* strain used as the donor is to be alpha toxin minus. It is suggested that, if possible, the donor *Staphylococcus aureus* strain should lack other toxins with LD_{50}s in the range of one microgram per kilogram body weight such as the exfoliative toxin.

Appendix F-IV-E. Fragments F-1, F-2, and F-3 of the diphtheria toxin gene (tox) may be cloned in *E. coli* K-12 under BL1+EK1 containment conditions and may be cloned in *Bacillus subtilis* host-vector systems under BL1 containment conditions. Fragment F-1 and fragment F-2 both contain: (i) Some or all of the transcriptional control elements of *tox;* (ii) the signal peptide; and (iii) fragment A (the center responsible for ADP-ribosylation of elongation factor 2). Fragment F-3 codes for most of the non-toxic fragment B of the toxin and contains no sequences coding for any portion of the enzymatically active fragment A moiety.

Appendix F–IV–F. The gene(s) coding for a toxin (designated LT-like) isolated from *E. coli* which is similar to the *E. coli* heat labile enterotoxin (LT) with respect to its activities and mode of action but is not neutralized by antibodies against cholera enterotoxin or against LT from human or porcine *E. coli* strains, and sequences homologous to the *E. coli* LT-like toxin gene may be cloned under BL1+EK1 conditions.

Appendix F–IV–G. Genes from *Vibrio fluvialis, Vibrio mimicus,* and non 0–1 *Vibrio cholerae,* specifying virulence factors for animals, may be cloned under BL1+EK1 conditions. The virulence factors to be cloned will be selected by testing fluid induction in suckling mice and Y–1 mouse adrenal cells.

Appendix F–IV–H. The intact structural gene(s) of the Shiga-like toxin from bacterial species classified in the families *Enterobacteriaceae* or *Vibrionaceae* including *Campylobacter* species may be cloned in *E. coli* K-12 under BL3+EK1 containment conditions.

E. coli host-vector systems expressing the Shiga-like toxin gene product may be moved from BL3+EK1 to BL2+EK1 containment conditions provided that: (1) The amount of toxin produced by the modified host-vector systems be no greater than that produced by the positive control strain *Shigella dysenteriae* 60R, grown and measured under optimal conditions; and (2) the cloning vehicle is to be an EK1 vector preferably belonging to the class of poorly mobilizable plasmids such as pBR322, pBR328, and pBR325.

Nontoxinogenic fragments of the Shiga-like toxin structural gene(s) may be moved from BL3+EK1 to BL2+EK1 containment conditions or such nontoxic fragments may be directly cloned in *E. coli* K–12 under BL2+EK1 conditions provided that the *E. coli* host-vector systems containing the fragments do not contain overlapping fragments which together would encompass the Shigalike toxin structural gene(s).

Appendix F–IV–I. A hybrid gene in which the gene coding for the melanocyte stimulating hormone (MSH) is joined to a segment of the gene encoding diphtheria toxin may be safely propagated in *E. coli* K–12 under BL4 containment in high containment building 550 at the Frederick Cancer Research Facility. If the investigators wish to proceed with the experiment, a prior review will be conducted to advise NIH whether the proposal has sufficient scientific merit to justify the use of the NIH BL4 facility. Before any of the strains may be removed from the BL4 facility, data on their safety shall be evaluated by the Working Group in Toxins and the working group recommendation shall be acted upon by NIH.

Appendix F–IV–J. The gene segment encoding the A subunit of chlolera toxin of *Vibrio cholerae* may be joined to the transposons Tn5 and Tn5–131 and the A-subunit::Tn5–131 hybrid gene cloned in *E. coli* K–12 and *V. cholerae* under BL1 containment conditions.

Appendix F–IV–K. A hybrid gene in which the gene coding for interleukin 2 (IL–2) is joined to a specific segment of the gene encoding diphtheria toxin may be propagated in *E. coli* K–12 host-vector systems under BL2 containment plus BL3 practices, with the use of poorly mobilizable plasmid vectors such as EK2 certified plasmids.

Appendix G—Physical Containment

Appendix G–I—Standard Practices and Training

The first principle of containment is a strict adherence to good microbiological practices [1–10]. Consequently, all personnel directly or indirectly involved in experiments on recombinant DNAs must receive adequate instruction (see Sections IV–B–1–e and IV–B–5–d). This shall, as a minimum, include instructions in aseptic techniques and in the biology of the organisms used in the experiments so that the potential biohazards can be understood and appreciated.

Any research group working with agents with a known or potential biohazard shall have an emergency plan which describes the procedures to be followed if an accident contaminates personnel or the environment. The PI must ensure that everyone in the laboratory is familiar with both the potential hazards of the work and the emergency plan (see Sections IV–B–3–d and IV–B–5–e). If a research group is working with a known pathogen for which there is an effective vaccine, the vaccine should be made available to all workers. Where serological monitoring is clearly appropriate, it shall be provided (see Section IV–B–1–f).

The "Laboratory Safety Monograph" and *Biosafety in Microbiological and Biomedical Laboratories* [2] booklets describe practices, equipment, and facilities in detail.

Appendix G–II—Physical Containment Levels

The objective of physical containment is to confine organisms containing recombinant DNA molecules and thus to reduce the potential for exposure of the laboratory worker, persons outside of the laboratory, and the environment to organisms containing recombinant DNA molecules. Physical containment is achieved through the use of laboratory practices, containment equipment, and special laboratory design. Emphasis is placed on primary means of physical containment which are provided by laboratory practices and containment equipment. Special laboratory design provides a secondary means of protection against the accidental release of organisms outside the laboratory or to the environment. Special laboratory design is used primarily in facilities in which experiments of moderate to high potential hazards are performed.

Combinations of laboratory practices, containment equipment, and special laboratory design can be made to achieve different levels of physical containment. Four levels of physical containment, which are designated as BL1, BL2, BL3, and BL4, are described. It should be emphasized that the descriptions and assignments of physical containment detailed below are based on existing approaches to containment of pathogenic organisms [2]. The National Cancer Institute describes three levels for research on oncogenic viruses which roughly correspond to our BL2, BL3, and BL4 level [3].

It is recognized that several different combinations of laboratory practices, containment equipment, and special laboratory design may be appropriate for containment of specific research activities. The Guidelines, therefore, allow alternative selections of primary containment equipment within facilities that have been designed to provide BL3 and BL4 levels of physical containment. The selection of alternative methods of primary containment is dependent, however, on the level of biological containment provided by the host-vector system used in the experiment. Consideration will also be given by the Director, NIH, with the advice of the RAC to other combinations which achieve an equivalent level of containment (see Section IV–C–1–b–(2)–(b)).

Appendix G–II–A—Biosafety Level 1 (BL1) [13]

Appendix G–II–A–1. Standard Microbiological Practices.

Appendix G–II–A–1–a. Access to the laboratory is limited or restricted at the discretion of the laboratory director when experiments are in progress.

Appendix G–II–A–1–b. Work surfaces are decontaminated once a day and after any spill of viable material.

Appendix G–II–A–1–c. All contaminated liquid or solid wastes are decontaminated before disposal.

Appendix G–II–A–1–d. Mechanical pipetting devices are used; mouth pipetting is prohibited.

Appendix G–II–A–1–e. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.

Appendix G–II–A–1–f. Persons wash their hands after they handle materials involving organisms containing recombinant DNA molecules, and animals, and before leaving the laboratory.

Appendix G–II–A–1–g. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G–II–A–1–h. It is recommended that laboratory coats, gowns, or uniforms be worn to prevent contamination or soiling of street clothes.

Appendix G–II–A–2—Special Practices

Appendix G–II–A–2–a. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leakproof container which is closed before being removed from the laboratory.

Appendix G–II–A–2–b. An insect and rodent control program is in effect.

Appendix G–II–A–3—Containment Equipment

Appendix G–II–A–3–a. Special containment equipment is generally not required for manipulations of agents assigned to Biosafety Level 1.

Appendix G–II–A–4—Laboratory Facilities

Appendix G–II–A–4–a. The laboratory is designed so that it can be easily cleaned.

Appendix G–II–A–4–b. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G–II–A–4–c. Laboratory furniture is sturdy. Spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G–II–A–4–d. Each laboratory contains a sink for hand-washing.

Appendix G–II–A–4–e. If the laboratory has windows that open, they are fitted with fly screens.

Appendix G–II–B—Biosafety Level 2 (BL2) [14]

Appendix G–II–B–1. Standard Microbiological Practices.

Appendix G–II–B–1–a. Access to the laboratory is limited or restricted by the laboratory director when work with organisms containing recombinant DNA molecules is in progress.

Appendix G–II–B–1–b. Work surfaces are decontaminated at least once a day and after any spill of viable material.

Appendix G–II–B–1–c. All contaminated liquid or solid wastes are decontaminated before disposal.

Appendix G–II–B–1–d. Mechanical pipetting devices are used; mouth pipetting is prohibited.

Appendix G–II–B–1–e. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.

Appendix G–II–B–1–f. Persons wash their hands after handling materials involving organisms containing recombinant DNA molecules, and animals, and when they leave the laboratory.

Appendix G–II–B–1–g. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G–II–B–1–h. Experiments of lesser biohazard potential can be carried out concurrently in carefully demarcated areas of the same laboratory.

Appendix G–II–B–2—Special Practices

Appendix G–II–B–2–a. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leakproof container which is closed before being removed from the laboratory.

Appendix G–II–B–2–b. The laboratory director limits access to the laboratory. The director has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory.

Appendix G–II–B–2–c. The laboratory director establishes policies and procedures whereby only persons who have been advised of the potential hazard and meet any specific entry requirements (e.g., immunization) enter the laboratory or animal rooms.

Appendix G–II–B–2–d. When the organisms containing recombinant DNA molecules in use in the laboratory require special provisions for entry (e.g., vaccination), a hazard warning sign incorporating the universal biohazard symbol is posted on the access door to the laboratory work area. The hazard warning sign identifies the agent, lists the name and telephone number of the laboratory director or other responsible person(s), and indicates the special requirement(s) for entering the laboratory.

Appendix G–II–B–2–e. An insect and rodent control program is in effect.

Appendix G–II–B–2–f. Laboratory coats, gowns, smocks, or uniforms are worn while in the laboratory. Before leaving the laboratory for nonlaboratory areas (e.g., cafeteria, library, administrative offices), this protective clothing is removed and left in the laboratory or covered with a clean coat not used in the laboratory.

Appendix G–II–B–2–g. Animals not involved in the work being performed are not permitted in the laboratory.

Appendix G–II–B–2–h. Special care is taken to avoid skin contamination with organisms containing recombinant DNA molecules; gloves should be worn when handling experimental animals and when skin contact with the agent is unavoidable.

Appendix G–II–B–2–i. All wastes from laboratories and animal rooms are appropriately decontaminated before disposal.

Appendix G–II–B–2–j. Hypodermic needles and syringes are used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) are used for the injection or aspiration of fluids containing organisms that contain recombinant DNA molecules. Extreme caution should be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Needles should not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe following use. The needle and syringe should be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix G–II–B–2–k. Spills and accidents which result in overt exposures to organisms containing recombinant DNA molecules are immediately reported to the laboratory director. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix G–II–B–2–l. When appropriate, considering the agent(s) handled, baseline serum samples for laboratory and other at-risk personnel are collected and stored. Additional serum specimens may be collected periodically depending on the agents handled or the function of the facility.

Appendix G–II–B–2–m. A biosafety manual is prepared or adopted. Personnel are advised of special hazards and are required to read

instructions on practices and procedures and to follow them.

Appendix G–II–B–3—Containment Equipment

Appendix G–II–B–3–a. Biological safety cabinets (Class I or II) (see Appendix G–III–12) or other appropriate personal protective or physical containment devices are used whenever:

Appendix G–II–B–3–a–(1). Procedures with a high potential for creating aerosols are conducted [15]. These may include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of materials whose internal pressures may be different from ambient pressures, inoculating animals intranasally, and harvesting infected tissues from animals or eggs.

Appendix G–II–B–3–a–(2). High concentrations or large volumes of organisms containing recombinant DNA molecules are used. Such materials may be centrifuged in the open laboratory if sealed heads or centrifuge safety cups are used and if they are opened only in a biological safety cabinet.

Appendix G–II–B–4—Laboratory Facilities

Appendix G–II–B–4–a. The laboratory is designed so that it can be easily cleaned.

Appendix G–II–B–4–b. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G–II–B–4–c. Laboratory furniture is sturdy and spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G–II–B–4–d. Each laboratory contains a sink for hand-washing.

Appendix G–II–B–4–e. If the laboratory has windows that open, they are fitted with fly screens.

Appendix G–II–B–4–f. An autoclave for decontaminating laboratory wastes is available.

Appendix G–II–C—Biosafety Level 3 (BL3) [16]

Appendix G–II–C–1. Standard Microbiological Practices.

Appendix G–II–C–1–a. Work surfaces are decontaminated at least once a day and after any spill of viable material.

Appendix G–II–C–1–b. All contaminated liquid or solid wastes are decontaminated before disposal.

Appendix G–II–C–1–c. Mechanical pipetting devices are used; mouth pipetting is prohibited.

Appendix G–II–C–1–d. Eating, drinking, smoking, storing food, and applying cosmetics are not permitted in the work area.

Appendix G–II–C–1–e. Persons wash their hands after handling materials involving organisms containing recombinant DNA molecules, and animals, and when they leave the laboratory.

Appendix G–II–C–1–f. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G–II–C–1–g. Persons under 16 years of age shall not enter the laboratory.

Appendix G–II–C–1–h. If experiments involving other organisms which require lower levels of containment are to be conducted in the same laboratory concurrently with experiments requiring BL3 level physical containment, they shall be conducted in accordance with all BL3 level laboratory practices.

Appendix G–II–C–2—Special Practices

Appendix G–II–C–2–a. Laboratory doors are kept closed when experiments are in progress.

Appendix G–II–C–2–b. Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leakproof container which is closed before being removed from the laboratory.

Appendix G–II–C–2–c. The laboratory director controls access to the laboratory and restricts access to persons whose presence is required for program or support purposes. The director has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory.

Appendix G–II–C–2–d. The laboratory director establishes policies and procedures whereby only persons who have been advised of the potential biohazard, who meet any specific entry requirements (e.g., immunization), and who comply with all entry and exit procedures enter the laboratory or animal rooms.

Appendix G–II–C–2–e. When organisms containing recombinant DNA molecules or experimental animals are present in the laboratory or containment module, a hazard warning sign incorporating the universal biohazard symbol is posted on all laboratory and animal room access doors. The hazard warning sign identifies the agent, lists the name and telephone number of the laboratory director or other responsible person(s), and indicates any special requirements for entering the laboratory, such as the need for immunizations, respirators, or other personal protective measures.

Appendix G–II–C–2–f. All activities involving organisms containing recombinant DNA molecules are conducted in biological safety cabinets or other physical containment devices within the containment module. No work in open vessels is conducted on the open bench.

Appendix G–II–C–2–g. The work surfaces of biological safety cabinets and other containment equipment are decontaminated when work with organisms containing recombinant DNA molecules is finished. Plastic-backed paper toweling used on nonperforated work surfaces within biological safety cabinets facilitates clean-up.

Appendix G–II–C–2–h. An insect and rodent program is in effect.

Appendix G–II–C–2–i. Laboratory clothing that protects street clothing (e.g., solid front or wrap-around gowns, scrub suits, coveralls) is worn in the laboratory. Laboratory clothing is not worn outside the laboratory, and it is decontaminated before being laundered.

Appendix G–II–C–2–j. Special care is taken to avoid skin contamination with contaminated materials; gloves should be worn when handling infected animals and when skin contact with infectious materials is unavoidable.

Appendix G–II–C–2–k. Molded surgical masks or respirators are worn in rooms containing experimental animals.

Appendix G–II–C–2–l. Animals and plants not related to the work being conducted are not permitted in the laboratory.

Appendix G–II–C–2–m. Laboratory animals held in a BL3 area shall be housed in partial-containment caging systems, such as Horsfall units [11], open cages placed in ventilated enclosures, solid-wall and -bottom cages covered by filter bonnets, or solid-wall and -bottom cages placed on holding racks equipped with ultraviolet in radiation lamps and reflectors.

Note.—Conventional caging systems may be used provided that all personnel wear appropriate personal protective devices. These shall include at a minimum wrap-around gowns, head covers, gloves, shoe covers, and respirators. All personnel shall shower on exit from areas where these devices are required.

Appendix G–II–C–2–n. All wastes from laboratories and animal rooms are appropriately decontaminated before disposal.

Appendix G–II–C–2–o. Vacuum lines are protected with high efficiency particulate air (HEPA) filters and liquid disinfectant traps.

Appendix G–II–C–2–p. Hypodermic needles and syringes are used only for parenteral injection and aspiration of fluids from laboratory animals and

diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) are used for the injection or aspiration of fluids containing organisms that contain recombinant DNA molecules. Extreme caution should be used when handling needles and syringes to avoid autoinoculation and the generation of aerosols during use and disposal. Needles should not be bent, sheared, replaced in the needle sheath or guard or removed from the syringe following use. The needle and syringe should be promptly placed in a puncture-resistant container and decontaminated, preferably by autoclaving, before discard or reuse.

Appendix G–II–C–2–q. Spills and accidents which result in overt or potential exposures to organisms containing recombinant DNA molecules are immediately reported to the laboratory director. Appropriate medical evaluation, surveillance, and treatment are provided and written records are maintained.

Appendix G–II–C–2–r. Baseline serum samples for all laboratory and other at-risk personnel should be collected and stored. Additional serum specimens may be collected periodically depending on the agents handled or the function of the laboratory.

Appendix G–II–C–2–s. A biosafety manual is prepared or adopted. Personnel are advised of special hazards and are required to read instructions on practices and procedures and to follow them.

Appendix G–II–C–2–t. Alternative Selection of Containment Equipment. Experimental procedures involving a host-vector system that provides a one-step higher level of biological containment than that specified can be conducted in the BL3 laboratory using containment equipment specified for the BL2 level of physical containment. Experimental procedures involving a host-vector system that provides a one-step lower level of biological containment than that specified can be conducted in the BL3 laboratory using containment equipment specified for the BL4 level of physical containment. Alternative combination of containment safeguards are shown in Table 1.

Appendix G–II–C–3—Containment Equipment

Appendix G–II–C–3–a. Biological safety cabinets (Class I, II, or III) (see Appendix G–III–12) or other appropriate combinations of personal protective or physical containment devices (e.g., special protective clothing, masks, gloves, respirators, centrifuge safety cups, sealed centrifuge rotors, and containment caging for animals) are used for all activities with organisms containing recombinant DNA molecules which pose a threat of aerosol exposure. These include: manipulation of cultures and of those clinical or environmental materials which may be a source of aerosols; the aerosol challenge of experimental animals; and harvesting infected tissues or fluids from experimental animals and embryonate eggs; and necropsy of experimental animals.

Appendix G–II–C–4—Laboratory Facilities

Appendix G–II–C–4–a. The laboratory is separated from areas which are open to unrestricted traffic flow within the building. Passage through two sets of doors is the basic requirement for entry into the laboratory from access corridors or other contiguous areas. Physical separation of the high containment laboratory from access corridors or other laboratories or activities may also be provided by a double-doored clothes change room (showers may be included), airlock, or other access facility which requires passage through two sets of doors before entering the laboratory.

Appendix G–II–C–4–b. The interior surfaces of walls, floors, and ceilings are water resistant so that they can be easily cleaned. Penetrations in these surfaces are sealed or capable of being sealed to facilitate decontaminating the area.

Appendix G–II–C–4–c. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G–II–C–4–d. Laboratory furniture is sturdy and spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G–II–C–4–e. Each laboratory contains a sink for hand-washing. The sink is foot, elbow, or automatically operated and is located near the laboratory exit door.

Appendix G–II–C–4–f. Windows in the laboratory are closed and sealed.

Appendix G–II–C–4–g. Access doors to the laboratory or containment module are self-closing.

Appendix G–II–C–4–h. An autoclave for decontaminating laboratory wastes is available preferably within the laboratory.

Appendix G–II–C–4–i. A ducted exhaust air ventilation system is provided. This system creates directional airflow that draws air into the laboratory through the entry area. The exhaust air is not recirculated to any other area of the building, is discharged to the outside, and is dispersed away from the occupied areas and air intakes. Personnel must verify that the direction of the airflow (into the laboratory) is proper. The exhaust air from the laboratory room can be discharged to the outside without being filtered or otherwise treated.

Appendix G–II–C–4–j. The HEPA-filtered exhaust air from Class I or Class II biological safety cabinets is discharged directly to the outside or through the building exhaust system. Exhaust air from Class I or II biological safety cabinets may be recirculated within the laboratory if the cabinet is tested and certified at least every twelve months. If the HEPA-filtered exhaust air from Class I or II biological safety cabinets is to be discharged to the outside through the building exhaust air system, it is connected to this system in a manner (e.g., thimble unit connection [12]) that avoids any interference with the air balance of the cabinets or building exhaust system.

Appendix G–II–D—Biosafety Level 4 (BL4).

Appendix G–II–D–1. Standard Microbiological Practices.

Appendix G–II–D–1–a. Work surfaces are decontaminated at least once a day and immediately after any spill of viable material.

Appendix G–II–D–1–b. Only mechanical pipetting devices are used.

Appendix G–II–D–1–c. Eating, drinking, smoking, storing food, and applying cosmetics are not permitted in the laboratory.

Appendix G–II–D–1–d. All procedures are performed carefully to minimize the creation of aerosols.

Appendix G–II–D–2—Special Practices

Appendix G–II–D–2–a. Biological materials to be removed from the Class III cabinets or from the maximum containment laboratory in a viable or intact state are transferred to a nonbreakable, sealed primary container and then enclosed in a nonbreakable, sealed secondary container which is removed from the facility through a disinfectant dunk tank, fumigation chamber, or an airlock designed for this purpose.

Appendix G–II–D–2–b. No materials, except for biological materials that are to remain in a viable or intact state, are removed from the maximum containment laboratory unless they have been autoclaved or decontaminated before they leave the facility. Equipment or material which might be damaged by high temperatures or steam is decontaminated by gaseous

or vapor methods in an airlock or chamber designed for this purpose.

Appendix G–II–D–2–c. Only persons whose presence in the facility or individual laboratory rooms is required for program or support purposes are authorized to enter. The supervisor has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory. Access to the facility is limited by means of secure, locked doors; accessibility is managed by the laboratory director, biohazards control officer, or other person responsible for the physical security of the facility. Before entering, persons are advised of the potential biohazards and instructed as to appropriate safeguards for ensuring their safety. Authorized persons comply with the instructions and all other applicable entry and exit procedures. A logbook signed by all personnel indicates the date and time of each entry and exit. Practical and effective protocols for emergency situations are established.

Appendix G–II–D–2–d. Personnel enter and leave the facility only through the clothing change and shower rooms. Personnel shower each time they leave the facility. Personnel use the airlocks to enter or leave the laboratory only in an emergency.

Appendix G–II–D–2–e. Street clothing is removed in the outer clothing change room and kept there. Complete laboratory clothing, including undergarments, pants and shirts or jumpsuits, shoes, and gloves, is provided and used by all personnel entering the facility. Head covers are provided for personnel who do not wash their hair during the exit shower. When leaving the laboratory and before proceeding into the shower area, personnel remove their laboratory clothing and store it in a locker or hamper in the inner change room.

Appendix G–II–D–2–f. When materials that contain organisms containing recombinant DNA molecules or experimental animals are present in the laboratory or animal rooms, a hazard warning sign incorporating the universal biohazard symbol is posted on all access doors. The sign identifies the agent, lists the name of the laboratory director or other responsible person(s), and indicates any special requirements for entering the area (e.g., the need for immunizations or respirators).

Appendix G–II–D–2–g. Supplies and materials needed in the facility are brought in by way of the double-doored autoclave, fumigation chamber, or airlock which is appropriately decontaminated between each use. After securing the outer doors, personnel within the facility retrieve the materials by opening the interior doors or the autoclave, fumigation chamber, or airlock. These doors are secured after materials are brought into the facility.

Appendix G–II–D–2–h. An insect and rodent control program is in effect.

Appendix G–II–D–2–i. Materials (e.g., plants, animals, and clothing) not related to the experiment being conducted are not permitted in the facility.

Appendix G–II–D–2–j. Hypodermic needles and syringes are used only for parenteral injection and aspiration of fluids from laboratory animals and diaphragm bottles. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral part of unit) are used for the injection or aspiration of fluids containing organisms that contain recombinant DNA molecules. Needles should not be bent, sheared, replaced in the needle sheath or guard, or removed from the syringe following use. The needle and syringe should be placed in a puncture-resistant container and decontaminated, preferably by autoclaving before discard or reuse. Whenever possible, cannulas are used instead of sharp needles (e.g., gavage).

Appendix G–II–D–2–k. A system is set up for reporting laboratory accidents and exposures and employee absenteeism and for the medical surveillance of potential laboratory-associated illnesses. Written records are prepared and maintained. An essential adjunct to such a reporting-surveillance system is the availability of a facility for quarantine, isolation, and medical care of personnel with potential or known laboratory associated illnesses.

Appendix G–II–D–2–l. Laboratory animals involved in experiments requiring BL4 level physical containment shall be housed either in cages contained in Class III cabinets or in partial containment caging systems (such as Horsfall units [11]), open cages placed in ventilated enclosures, or solid-wall and -bottom cages placed on holding racks equipped with ultraviolet irradiation lamps and reflectors that are located in a specially designed area in which all personnel are required to wear one-piece positive pressure suits.

Appendix G–II–D–2–m. Alternative Selection of Containment Equipment. Experimental procedures involving a host-vector system that provides a one-step higher level of biological containment than that specified can be conducted in the BL4 facility using containment equipment requirements specified for the BL3 level of physical containment. Alternative combinations of containment safeguards are shown in Table I.

Appendix G–II–D–3.—Containment Equipment

Appendix G–II–D–3–a. All procedures within the facility with agents assigned to Biosafety Level 4 are conducted in the Class III biological safety cabinet or in Class I or II biological safety cabinets used in conjunction with one-piece positive pressure personnel suits ventilated by a life-support system.

Appendix G–II–D–4.—Laboratory Facilities

Appendix G–II–D–4–a. The maximum containment facility consists of either a separate building or a clearly demarcated and isolated zone within a building. Outer and inner change rooms separated by a shower are provided for personnel entering and leaving the facility. A double-doored autoclave, fumigation chamber, or ventilated airlock is provided for passage of those materials, supplies, or equipment which are not brought into the facility through the change room.

Appendix G–II–D–4–b. Walls, floors, and ceilings of the facility are constructed to form a sealed internal shell which facilitates fumigation and is animal and insect proof. The internal surfaces of this shell are resistant to liquids and chemicals, thus facilitating cleaning and decontamination of the area. All penetrations in these structures and surfaces are sealed. Any drains in the floors contain traps filled with a chemical disinfectant of demonstrated efficacy against the target agent, and they are connected directly to the liquid waste decontamination system. Sewer and other ventilation lines contain HEPA filters.

Appendix G–II–D–4–c. Internal facility appurtenances, such as light fixtures, air ducts, and utility pipes, are arranged to minimize the horizontal surface area on which dust can settle.

Appendix G–II–D–4–d. Bench tops have seamless surfaces which are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.

Appendix G–II–D–4–e. Laboratory furniture is of simple and sturdy construction, and spaces between benches, cabinets, and equipment are accessible for cleaning.

Appendix G–II–D–4–f. A foot, elbow, or automatically operated hand-washing sink is provided near the door of each laboratory room in the facility.

Appendix G–II–D–4–g. If there is a central vacuum system, it does not serve areas outside the facility. In-line HEPA filters are placed as near as practicable to each use point or service cock. Filters

are installed to permit in-place decontamination and replacement. Other liquid and gas services to the facility are protected by devices that prevent backflow.

Appendix G–II–D–4–h. If water fountains are provided, they are foot operated and are located in the facility corridors outside the laboratory. The water service to the fountain is not connected to the backflow-protected distribution system supplying water to the laboratory areas.

Appendix G–II–D–4–i. Access doors to the laboratory are self-closing and lockable.

Appendix G–II–D–4–j. Any windows are breakage resistant.

Appendix G–II–D–4–k. A double-doored autoclave is provided for decontaminating materials passing out of the facility. The autoclave door which opens to the area external to the facility is sealed to the outer wall and automatically controlled so that the outside door can only be opened after the autoclave "sterilization" cycle has been completed.

Appendix G–II–D–4–l. A pass-through dunk tank, fumigation chamber, or an equivalent decontamination method is provided so that materials and equipment that cannot be decontaminated in the autoclave can be safely removed from the facility.

Appendix G–II–D–4–m. Liquid effluents from laboratory sinks, biological safety cabinets, floors, and autoclave chambers are decontaminated by heat treatment before being released from the maximum containment facility. Liquid wastes from shower rooms and toilets may be decontaminated with chemical disinfectants or by heat in the liquid waste decontamination system. The procedure used for heat decontamination of liquid wastes is evaluated mechanically and biologically by using a recording thermometer and an indicator microorganism with a defined heat susceptibility pattern. If liquid wastes from the shower room are decontaminated with chemical disinfectants, the chemical used is of demonstrated efficacy against the target or indicator microorganisms.

Appendix G–II–D–4–n. An individual supply and exhaust air ventilation system is provided. The system maintains pressure differentials and directional airflow as required to assure flows inward from areas outside of the facility toward areas of highest potential risk within the facility. Manometers are used to sense pressure differentials between adjacent areas maintained at different pressure levels. If a system malfunctions, the manometers sound an alarm. The supply and exhaust airflow is interlocked to assure inward (or zero) airflow at all times.

Appendix G–II–D–4–o. The exhaust air from the facility is filtered through HEPA filters and discharged to the outside so that it is dispersed away from occupied buildings and air intakes. Within the facility, the filters are located as near the laboratories as practicable in order to reduce the length of potentially contaminated air ducts. The filter chambers are designed to allow *in situ* decontamination before filters are removed and to facilitate certification testing after they are replaced. Coarse filters and HEPA filters are provided to treat air supplied to the facility in order to increase the lifetime of the exhaust HEPA filters and to protect the supply air system should air pressures become unbalanced in the laboratory.

Appendix G–II–D–4–p. The treated exhaust air from Class I and II biological safety cabinets can be discharged into the laboratory room environment or the outside through the facility air exhaust system. If exhaust air from Class I or II biological safety cabinets is discharged into the laboratory the cabinets are tested and certified at 6-month intervals. *The exhaust air from Class III biological safety cabinets is discharged, without recirculation through two sets of HEPA filters in series, via the facility exhaust air system.* If the treated exhaust air from any of these cabinets is discharged to the outside through the facility exhaust air system, it is connected to this system in a manner (e.g., thimble unit connection [12]) that avoids any interference with the air balance of the cabinets or the facility exhaust air system.

Appendix G–II–D–4–q. A specially designed suit area may be provided in the facility. Personnel who enter this area wear a one-piece positive pressure suit that is ventilated by a life-support system. The life-support system includes alarms and emergency backup breathing air tanks. Entry to this area is through an airlock fitted with airtight doors. A chemical shower is provided to decontaminate the surface of the suit before the worker leaves the area. The exhaust air from the suit area is filtered by two sets of HEPA filters installed in series. A duplicate filtration unit, exhaust fan, and an automatically starting emergency power source are provided. The air pressure within the suit area is lower than that of any adjacent area. Emergency lighting and communication systems are provided. All penetrations into the internal shell of the suit area are sealed. A double-doored autoclave is provided for decontaminating waste materials to be removed from the suit area.

TABLE 1.—POSSIBLE ALTERNATE COMBINATIONS OF PHYSICAL AND BIOLOGICAL CONTAINMENT SAFEGUARDS

Classification of physical and biological containment	Alternate physical containment			Alternate biological containment
	Laboratory facilities	Laboratory practices	Containment equipment	
BL3/HV2	BL3	BL3	BL3	HV2
	BL3	BL3	BL4	HV1
BL3/HV1	BL3	BL3	BL3	HV1
	BL3	BL3	BL2	HV2
BL4/HV1	BL4	BL4	BL4	HV1
	BL4	BL4	BL3	HV2

Appendix G–III—Footnotes and References of Appendix G

1. *Laboratory Safety at the Center for Disease Control* (Sept. 1974). U.S. Department of Health Education and Welfare Publication No. CDC 75–8118.

2. *Biosafety in Microbiological and Biomedical Laboratories*, 1st Edition (March 1984), U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Georgia 30333, and National Institutes of Health, Bethesda, Maryland 20205.

3. *National Cancer Institute Safety Standards for Research Involving Oncogenic Viruses* (Oct. 1974). U.S. Department of Health, Education and Welfare Publication No. (NIH) 75–790.

4. *National Institutes of Health Biohazards Safety Guide* (1974). U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health. U.S. Government Printing Office, Stock No. 1740–00383.

5. *Biohazards in Biological Research* (1973). A. Hellman, M.N. Oxman, and R. Pollack (ed.) Cold Spring Harbor Laboratory.

6. *Handbook of Laboratory Safety* (1971). 2nd Edition. N.V. Steere (ed.). The Chemical Rubber Co., Cleveland.

7. Bodily, J.L. (1970). *General Administration of the Laboratory*, H.L. Bodily, E.L. Updyke, and J.O. Mason (eds.), Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections. American Public Health Association, New York, pp. 11–28.

8. Darlow, H.M. (1969). *Safety in the Microbiological Laboratory*. In J.R. Norris and D.W. Robbins (ed.), Methods in Microbiology. Academic Press, Inc., New York, pp. 169–204.

9. *The Prevention of Laboratory Acquired Infection* (1974). C.H. Collins, E.G. Hartley, and R. Pilsworth. Public Health Laboratory Service, Monograph Series No. 6.

10. Chatigny, M.A. (1961). *Protection Against Infection in the Microbiological Laboratory: Devices and Procedures*. In W.W. Umbreit (ed.). Advances in Applied Microbiology. Academic Press, New York, N.Y. 3:131–192.

11. Horsfall, F.L., Jr., and J.H. Baner (1940). *Individual Isolation of Infected Animals in a Single Room*. J. Bact. *40*, 569–580.

12. Biological safety cabinets referred to in this section are classified as *Class I, Class II,* or *Class III* cabinets. A *Class I* is a ventilated cabinet for personnel protection having an

inward flow of air away from the operator. The exhaust air from this cabinet is filtered through a high-efficiency particulate air (HEPA) filter. This cabinet is used in three operational modes: (1) with a full-width open front, (2) with an installed front closure panel (having four 8-inch diameter openings) without gloves, and (3) with an installed front closure panel equipped with arm-length rubber gloves. The face velocity of the inward flow of air through the full-width open front is 75 feet per minute or greater.

A *Class II* cabinet is a ventilated cabinet for personnel and product protection having an open front with inward air flow for personnel protection, and HEPA filtered mass recirculated air flow for product protection. The cabinet exhaust air is filtered through a HEPA filter. The face velocity of the inward flow of air through the full-width open front is 75 feet per minute or greater. Design and performance specifications for *Class II* cabinets have been adopted by the National Sanitation Foundation, Ann Arbor, Michigan. A *Class III* cabinet is a closed-front ventilated cabinet of gas-tight construction which provides the highest level of personnel protection of all biohazard safety cabinets. The interior of the cabinet is protected from contaminants exterior to the cabinet. The cabinet is fitted with arm-length rubber gloves and is operated under a negative pressure of at least 0.5 inches water gauge. All supply air is filtered through HEPA filters. Exhaust air is filtered through two HEPA filters or one HEPA filter and incinerator before being discharged to the outside environment. National Sanitation Foundation Standard 49. 1976. Class II (Laminar Flow) Biohazard Cabinetry. Ann Arbor, Michigan.

13. Biosafety Level 1 is suitable for work involving agents of no known or minimal potential hazard to laboratory personnel and the environment. The laboratory is not separated from the general traffic patterns in the building. Work is generally conducted on open bench tops. Special containment equipment is not required or generally used. Laboratory personnel have specific training in the procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology or a related science (see Appendix G–III–2).

14. Biosafety Level 2 is similar to Level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment. It differs in that: (1) laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists; (2) access to the laboratory is limited when work is being conducted; and (3) certain procedures in which infectious aerosols are created are conducted in biological safety cabinets or other physical containment equipment (see Appendix G–III–2).

15. Office of Research Safety, National Cancer Institute, and the Special Committee of Safety and Health Experts. 1978. "Laboratory Safety Monograph: A Supplement to the NIH Guidelines for Recombinant DNA Research." Bethesda, Maryland, National Institutes of Health.

16. Biosafety Level 3 is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Laboratory personnel have specific training in handling pathogenic and potentially lethal agents and are supervised by competent scientists who are experienced in working with these agents. All procedures involving the manipulation of infectious material are conducted within biological safety cabinets or other physical containment devices or by personnel wearing appropriate personal protective clothing and devices. The laboratory has special engineering and design features. It is recognized, however, that many existing facilities may not have all the facility safeguards recommended for Biosafety Level 3 (e.g., access zone, sealed penetrations, and directional airflow, etc.). In these circumstances, acceptable safety may be achieved for routine or repetitive operations (e.g., diagnostic procedures involving the propagation of an agent for identification, typing, and susceptibility testing) in laboratories where facility features satisfy Biosafety Level 2 recommendations provided the recommended "Standard Microbiological Practices," "Special Practices," and "Containment Equipment" for Biosafety Level 3 are rigorously followed. The decision to implement this modification of Biosafety Level 3 recommendations should be made only by the laboratory director (see Appendix G–III–2).

Appendix H—Shipment

Recombinant DNA molecules contained in an organism or virus shall be shipped only as an etiologic agent under requirements of the U.S. Public Health Service, and the U.S. Department of Transportation (§ 72.3, Part 72, Title 42, and §§ 173.386–.388, Part 173, Title 49, U.S. Code of Federal Regulations (CFR)) as specified below:

Appendix H–I

Recombinant DNA molecules contained in an organism or virus requiring BL1, BL2, or BL3 physical containment, when offered for transportation or transported, are subject to all requirements of §§ 72.3(a)–(e), Part 72, Title 42 CFR, and §§ 173.386–.388, Part 173, Title 49 CFR.

Appendix H–II

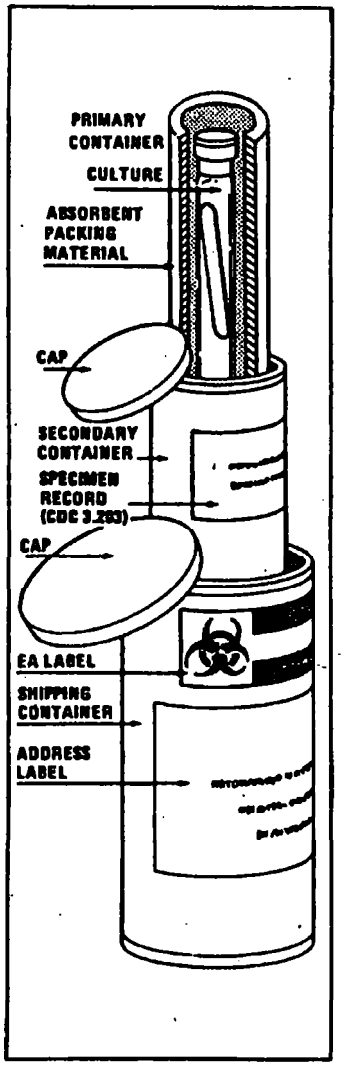
Recombinant DNA molecules contained in an organism or virus requiring BL4 physical containment, when offered for transportation or transported, are subject to the requirements listed above under Appendix H–I and are also subject to § 72.3(f), Part 72, Title 42 CFR.

Appendix H–III

Information on packaging and labeling of etiologic agents is shown in Figures 1, 2, and 3. Additional information on packaging and shipment is given in the "Laboratory Safety Monograph—A Supplement to the NIH Guidelines for Recombinant DNA Research," available from the Office of Recombinant DNA Activities and in *Biosafety in Microbiological and Biomedical Laboratories* (see Appendix G–III–2).

BILLING CODE 4140-01-M

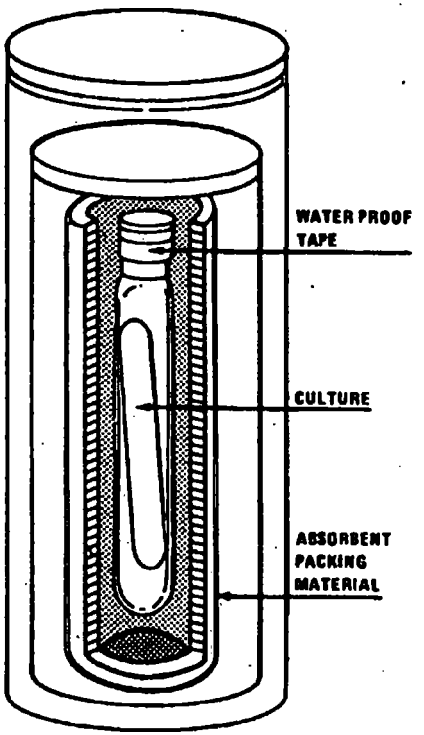
FIGURE 1



BILLING CODE 4140-01-C

PACKAGING AND LABELING OF ETIOLOGIC AGENTS

FIGURE 2



CROSS SECTION OF PROPER PACKING

The Interstate Shipment of Etiologic Agents (42 CFR, Part 72) was revised July 21, 1980 to provide for packaging and labeling requirements for etiologic agents and certain other materials shipped in interstate traffic.

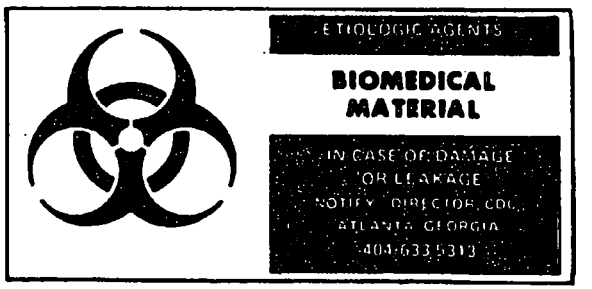
Figures 1 and 2 diagram the packaging and labeling of etiologic agents in volumes of less than 50 ml. in accordance with the provisions of subparagraph 72.3 (a) of the cited regulation. Figure illustrates the color and size of the label, described in subparagraph 72.3 (d) (1 - 5) of the regulations, which shall be affixed to all shipments of etiologic agents.

For further information on any provision of this regulation contact:

Centers for Disease Control
Attn: Biohazards Control Office
1600 Clifton Road
Atlanta, Georgia 30333

Telephone: 404–329-3883
FTS–236-3883

FIGURE 3



Appendix I—Biological Containment

(See also Appendix E)

Appendix I–I—Levels of Biological Containment.

In consideration of biological containment, the vector (plasmid, organelle, or virus) for the recombinant DNA and the host (bacterial, plant, or animal cell) in which the vector is propagated in the laboratory will be considered together. Any combination of vector and host which is to provide biological containment must be chosen or constructed so that the following types of "escape" are minimized: (i) Survival of the vector in its host outside the laboratory, and (ii) transmission of the vector from the propagation host to other nonlaboratory hosts.

The following levels of biological containment (HV, or *Host-Vector*, systems) for prokaryotes will be established; specific criteria will depend on the organisms to be used.

Appendix I–I–A. HV1. A host-vector system which provides a moderate level of containment. *Specific systems* are:

Appendix I–I–A–1. EK1. The host is always *E. coli* K–12 or a derivative thereof, and the vectors include nonconjugative plasmids (e.g., pSC101, ColEl, or derivatives thereof [1–7] and variants of bacteriophage, such as lambda [8–15]. The *E. coli* K–12 hosts shall not contain conjugation-proficient plasmids, whether autonomous or integrated, or generalized transducing phages.

Appendix I–I–A–2. Other HV1. Hosts and vectors shall be, at a minimum, comparable in containment to *E. coli* K–12 with a non conjugative plasmid or bacteriophage vector. The data to be considered and a mechanism for approval of such HV1 systems are described below (Appendix I–II).

Appendix I–I–B. HV2. These are host-vector systems shown to provide a high level of biological containment as demonstrated by data from suitable tests performed in the laboratory. Escape of the recombinant DNA either via survival of the organisms or via transmission of recombinant DNA to other organisms should be less than $1/10^8$ under specified conditions. Specific systems are:

Appendix I–I–B–1. For EK2 host-vector systems in which the vector is a plasmid, no more than one in 10^8 host cells should be able to perpetuate a cloned DNA fragment under the specified nonpermissive laboratory conditions designed to represent the natural environment, either by survival of the original host or as a consequences of transmission of the cloned DNA fragment.

Appendix I–I–B–2. For EK2 host-vector systems in which the vector is a phage, no more than one in 10^8 phage particles should be able to perpetuate a cloned DNA fragment under the specified nonpermissive laboratory conditions designed to represent the natural environment either: (i) as a prophage (in the inserted or plasmid form) in the laboratory host used for phage propagation or (ii) by surviving in natural environments and transferring a cloned DNA fragment to other hosts (or their resident prophages).

Appendix I–II—Certification of Host-Vector Systems

Appendix I–II–A. Responsibility. HV1 systems other than *E. coli* K–12 and HV2 host-vector systems may not be designated as such until they have been certified by the Director, NIH. Application for certification of a host-vector system is made by written application to the Office of Recombinant DNA Activities, National Institutes of Health, Building 31, Room 3B10, Bethesda, Maryland 20892.

Host-vector systems that are proposed for certification will be reviewed by the RAC (see Section IV–C–1–b–(1)–(e)). This will first involve review of the data on construction, properties, and testing of the proposed host-vector system by a working group composed of one or more members of the RAC and other persons chosen because of their expertise in evaluating such data. The committee will then evaluate the report of the working group and any other available information at a regular review meeting. The Director, NIH, is responsible for certification after receiving the advice of the RAC. Minor modifications of existing certified host-vector systems where the modifications are of minimal or no consequence to the properties relevant to containment may be certified by the Director, NIH, without review by the RAC (see Section IV–C–1–b–(3)–(c)).

When new host-vector systems are certified, notice of the certification will be sent by ORDA to the applicant and to all IBCs and will be published in the *Recombinant DNA Technical Bulletin.* Copies of a list of all currently certified host-vector systems may be obtained from ORDA at any time.

The Director, NIH, may at any time rescind the certification of any host-vector system (see Section IV–C–1–b–(3)–(d)). If certification of a host-vector system is rescinded, NIH will instruct investigators to transfer cloned DNA into a different system or use the clones at a higher physical containment level unless NIH determines that the already constructed clones incorporate adequate biological containment.

Certification of a given system does not extend to modifications of either the host or vector component of that system. Such modified systems must be independently certified by the Director, NIH. If modifications are minor, it may only be necessary for the investigator to submit data showing that the modifications have either improved or not impaired the major phenotypic traits on which the containment of the system depends. Substantial modifications of a certified system require the submission of complete testing data.

Appendix I–II–B. Data to be Submitted for Certification.

Appendix I–II–B–1. HV1 Systems Other than E. coli K–12. The following types of data shall be submitted, modified as appropriate for the particular system under consideration: (i) A description of the organism and vector; the strain's natural habitat and growth requirements; its physiological properties, particularly those related to its reproduction and survival and the mechanisms by which it exchanges genetic information; the range of organisms with which this organism normally exchanges genetic information and what sort of information is exchanged; and any relevant information on its pathogenicity or toxicity; (ii) a description of the history of the particular strains and vectors to be used, including data on any mutations which render this organism less able to survive or transmit genetic information; and (iii) a general description of the range of experiments contemplated with emphasis on the need for developing such an HV1 system.

Appendix I–II–B–2. HV2 Systems. Investigators planning to request HV2 certification for host-vector systems can obtain instructions from ORDA concerning data to be submitted [14–15]. In general, the following types of data are required: (i) Description of construction steps with indication of source, properties, and manner of introduction of genetic traits; (ii) quantitative data on the stability of genetic traits that contribute to the containment of the system; (iii) data on the survival of the host-vector system under nonpermissive laboratory conditions designed to represent the relevant natural environment; (iv) Data on transmissibility of the vector and/or a cloned DNA fragment under both permissive and nonpermissive conditions; (v) data on all other properties of the system which affect containment and utility, including

information on yields of phage or plasmid molecules, ease of DNA isolation, and ease of transfection or transformation; and (vi) in some cases, the investigator may be asked to submit data on survival and vector transmissibility from experiments in which the host-vector is fed to laboratory animals and human subjects. Such *in vivo* data may be required to confirm the validity of predicting *in vivo* survival on the basis of *in vitro* experiments.

Data must be submitted in writing to ORDA. Ten to twelve weeks are normally required for review and circulation of the data prior to the meeting at which such data can be considered by the RAC. Investigators are encouraged to publish their data on the construction, properties, and testing of proposed HV2 systems prior to consideration of the system by the RAC and its subcommittee. More specific instructions concerning the type of data to be submitted to NIH for proposed EK2 systems involving either plasmids or bacteriophage in *E. coli* K-12 are available from ORDA.

Appendix I-III—Footnotes and References of Appendix I

1. Hersfield, V., H.W. Boyer, C. Yanofsky, M.A. Lovett, and D.R. Helinski (1974). *Plasmid ColEI as a Molecular Vehicle for Cloning and Amplification of DNA.* Proc. Nat. Acad. Sci. USA *71*, 3455–3459.
2. Wensink, P.C., D.J. Finnegan, J.E. Donelson, and D.S. Hogness (1974). *A System for Mapping DNA Sequences in the Chromosomes of Drosophila Melanogaster.* Cell *3*, 315–335.
3. Tanaka, T., and B. Weisblum (1975). *Construction of a Colicin EI-R Factor Composite Plasmid In Vitro: Means for Amplification of Deoxyribonucleic Acid.* J. Bacteriol. *121*, 354–362.
4. Armstrong, K.A., V. Hershfield, and D.R. Helinski (1977). *Gene Cloning and Containment Properties of Plasmid Col EI and Its Derivatives*, Science *196*, 172–174.
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6. Cohen, S.N., A.C.W. Chang, H. Boyer, and R. Helling (1973). *Construction of Biologically Functional Bacterial Plasmids in Vitro.* Proc. Natl. Acad. Sci. USA *70*, 3240–3244.
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8. Thomas, M., J.R. Cameron, and R.W. Davis (1974). *Viable Molecular Hybrids of Bacteriophage Lambda and Eukaryotic DNA.* Proc. Nat. Acad. Sci. USA *71*, 4579–4583.
9. Murray, N.E., and K. Murray (1974). *Manipulation of Restriction Targets in Phage Lambda to Form Receptor Chromosomes for DNA Fragments.* Nature *251*, 476–481.
10. Ramback, A., and P. Tiollais (1974). *Bacteriophage Having EcoRI Endonuclease Sites Only in the Non-Essential Region of the Genome.* Proce. Nat. Acad. Sci., USA *71*, 3927–3930.
11. Blattner, F.R., B.G. Williams, A.E. Bleche, K. Denniston-Thompson, H.E. Faber, L.A. Furlong, D.J. Gunwald, D.O. Kiefer, D.D. Moore, J.W. Shumm, E.L. Sheldon, and O. Smithies (1977). *Charon Phages: Safer Derivatives of Bacteriophge Lambda for DNA Cloning.* Science *196*, 163–169.
12. Donoghue, D.J., and P.A. Sharp (1977). *An Improved Lambda Vector: Construction of Model Recombinants Coding for Kanamycin Resistance*, Gene *1*, 209–227.
13. Leder, P., D. Tiemeier and L. Enquist (1977). *EK2 Derivatives of Bacteriophage Lambda Useful in the Cloning of DNA from Higher Organisms: The λgt WES System.* Science *196*, 175–177.
14. Skalka, A. (1978). *Current Status of Coliphage λ EK2 Vectors.* Gene *3*, 29–35.
15. Szybalski, W., A. Skalka, S. Gottesman, A. Campbell, and D. Botstein (1978). *Standardized Laboratory Tests for EK2 Certification.* Gene *3*, 36–38.

Appendix J—Biotechnology Science Coordinating Committee

The following excerpts from its charter (signed October 30, 1985) describe the Biotechnology Science Coordinating Committee:

Purpose

The Domestic Policy Working Group on Biotechnology has determined that in the area of biotechnology with its rapid growth of scientific discovery, scientific issues of interagency concern will arise frequently and need to be communicated among the various agencies involved with reviews of biotechnology applications. The Federal Coordinating Council for Science, Engineering, and Technology (FCCSET) established by 42 U.S.C. 6651 is an interagency science committee chaired by the Director of the Office of Science and Technology Policy with the mission of coordinating science activities affecting more than one agency. Committees may be established under FCCSET for addressing particular science issues. Thus, the Biotechnology Science Coordinating Committee (BSCC) is established to provide formally an opportunity for interagency science policy coordination and guidance and for the exchange of information regarding the scientific aspects of biotechnology applications submitted to federal research and regulatory agencies for approval.

Functions

The BSCC will coordinate interagency review of scientific issues related to the assessments and approval of biotechnology research applications and biotechnology product applications and postmarketing surveillance when they involve the use of recombinant RNA, recombinant DNA, cell fusion or similar techniques.

The BSCC will:

(a) Serve as a coordinating forum for addresssing scientific problems, sharing information, and developing consensus;

(b) Promote consistency in the development of Federal agencies' review procedures and assessments;

(c) Facilitate continuing cooperation among Federal agencies on emerging scientific issues; and

(d) Identify gaps in scientific knowledge.

Authority

To accomplish these functions the BSCC is authorized to:

(a) Receive documentation from agencies necessary for the performance of its function;

(b) Conduct analyses of broad scientific issues that extend beyond those of any one agency;

(c) Develop generic scientific recommendations that can be applied to similar, recurring applications;

(d) Convene workshops, symposia, and generic research projects related to scientific issues in biotechnology; and

(e) Hold periodic public meetings.

Members and Chairman

The BSCC includes the following initial members:

Department of Agriculture
- Assistant Secretary for Marketing and Inspection Services
- Assistant Secretary for Science and Education

Department of Health and Human Services
- Commissioner, Food and Drug Administration
- Director, National Institutes of Health

Environmental Protection Agency
- Assistant Administrator for Pesticides and Toxic Substances
- Assistant Administrator for Research and Development

National Science Foundation
- Assistant Director of Biological, Behavorial & Social Sciences

The BSCC is chaired by the Assistant Director for Biological, Behavioral and Social Sciences of the National Science Foundation and the Director of the National Institutes of Health on a rotating basis.

Administrative Provisions

(a) The BSCC will report to the FCCSET through the Chair.

(b) Meetings of the BSCC shall be held periodically. Some public meetings will be held.

(c) Confidential business information and proprietary information shall be protected under the confidentiality requirements of each member agency.

(d) Subcommittees and working groups, with participation not restricted to BSCC members or full-time Federal employees, may be formed to assist the BSCC in its work.

(e) All BSCC members will be full-time Federal employees whose compensation, reimbursement for travel expenses and other costs shall be borne by their respective agencies.

(f) Each member of the BSCC shall provide such agency support and resources as may be available and necessary for the operation of the BSCC including undertaking special studies as come within the functions assigned herein.

(g) An Office of Science and Technology Policy staff member will serve as BSCC Executive Secretary.

Appendix K—Physical Containment for Large-Scale Uses of Organisms Containing Recombinant DNA Molecules

This part of the Guidelines specifices physical containment guidelines for large-scale (greater than 10 liters of culture) research or production involving viable organisms containing recombinant DNA molecules. It shall apply to large-scale research or production activities as specified in Section III–B–5 of the Guidelines.

All provisions of the Guidelines shall apply to large-scale research or production activities with the following modifications:

- Appendix K shall replace Appendix G when quantities in excess of 10 liters of culture are involved in research or production.
- The institutions shall appoint a Biological Safety Officer (BSO) if it engages in large-scale research or production activities involving viable organisms containing recombinant DNA molecules. The duties of the BSO shall include those specified in Section IV–B–4 of the Guidelines.
- The institution shall establish and maintain a health surveillance program for personnel engaged in large-scale research or production activities involving viable organisms containing recombinant DNA molecules which require BL3 containment at the laboratory scale. The program shall include: preassignment and periodic physical and medical examinations; collection, maintenance and analysis of serum specimens for monitoring serologic changes that may result from the employee's work experience; and provisions for the investigation of any serious, unusual or extended illnesses of employees to determine possible occupational origin.

Appendix K–I.—Selection of Physical Containment Levels.

The selection of the physical containment level required for recombinant DNA research or production involving more than 10 liters of culture is based on the containment guidelines established in Part III of the Guidelines. For purposes of large-scale research or production, three physical containment levels are established. These are referred to as BL1–LS, BL2–LS, and BL3–LS. The BL–LS level of physical containment is required for large-scale research or production of viable organisms containing recombinant DNA molecules which require BLI containment at the laboratory scale. (The BL1–LS level of physical containment is recommended for large-scale research or production of viable organisms for which BLI is recommended at the laboratory scale such as those described in Appendix C.) The BL2–LS level of physical containment is required for large-scale research or production of viable organisms containing recombinant DNA molecules which require BL2 containment at the laboratory scale. The BL3–LS level of physical containment is required for large-scale research or production of viable organisms containing recombinant DNA molecules which require BL3 contaiment at the laboratory scale. No provisions are made for large-scale research or production of viable organisms containing recombinant DNA molecules which require BL4 containment at the laboratory scale. If necessary, these requirements will be established by NIH on an individual basis.

Appendix K–II—BL1–LS Level

Appendix K–II–A. Cultures of viable organisms containing recombinant DNA molecules shall be handled in a closed system (e.g., closed vessel used for the propagation and growth of cultures) or other primary containment equipment (e.g., biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to reduce the potential for escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system or other primary containment equipment provided all physical containment requirements specified in Appendix G–II–A of the Guidelines are met.

Appendix K–II–B. Culture fluids (except as allowed in Appendix K–II–C) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant DNA molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant DNA molecules.

Appendix K–II–C. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of culture fluids from one closed system to another shall be done in a manner which minimizes the release of aerosols or contamination of exposed surfaces.

Appendix K–II–D. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to HEPA filters or by other equivalent procedures (e.g., incineration) to minimize the release of viable organisms containing recombinant DNA molecules to the environment.

Appendix K–II–E. A closed system or other primary containment equipment that has contained viable organisms containing recombinant DNA molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure. A validated sterilization procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant DNA molecules.

Appendix K–II–F. Emergency plans required by Section IV–B–3–f shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K–III—BL2–LS Level

Appendix K–III–A. Cultures of viable organisms containing recombinant DNA molecules shall be handled in a closed system (e.g., closed vessel used for the propagation and growth of cultures) or other primary containment equipment (e.g., Class III biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to prevent the escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system or other primary containment equipment provided all physical containment requirements specified in Appendix G–II–B of the Guidelines are met.

Appendix K–III–B. Culture fluids (except as allowed in Appendix K–III–C)

shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant DNA molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant DNA molecules.

Appendix K–III–C. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of cultures fluids from one closed system to another shall be done in a manner which prevents the release of aerosols or contamination of exposed surfaces.

Appendix K–III–D. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to HEPA filters or by other equivalent procedures (e.g., incineration) to prevent the release of viable organisms containing recombinant DNA molecules to the environment.

Appendix K–III–E. A closed system or other primary containment equipment that has contained viable organisms containing recombinant DNA molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure. A validated sterilization procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant DNA molecules.

Appendix K–III–F. Rotating seals and other mechanical devices directly associated with a closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be designed to prevent leakage or shall be fully enclosed in ventilated housings that are exhausted through filters which have efficiencies equivalent to HEPA filters or through other equivalent treatment devices.

Appendix K–III–G. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules and other primary containment equipment used to contain operations involving viable organisms containing recombinant DNA molecules shall include monitoring or sensing devices that monitor the integrity of containment during operations.

Appendix K–III–H. A closed system used for the propagation and growth of viable organisms containing the recombinant DNA molecules shall be tested for integrity of the containment features using the organism that will serve as the host for propagating recombinant DNA molecules. Testing shall be accomplished prior to the introduction of viable organisms containing recombinant DNA molecules and following modification or replacement of essential containment features. Procedures and methods used in the testing shall be appropriate for the equipment design and for recovery and demonstration of the test organism. Records of tests and results shall be maintained on file.

Appendix K–III–I. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be permanently identified. This identification shall be used in all records reflecting testing, operation, and maintenance and in all documentation relating to use of this equipment for research or production activities involving viable organisms containing recombinant DNA molecules.

Appendix K–III–J. The universal biohazard sign shall be posted on each closed system and primary containment equipment when used to contain viable organisms containing recombinant DNA molecules.

Appendix K–III–K. Emergency plans required by Section IV–B–3–f shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K–IV—BL3–LS Level

Appendix K–IV–A. Cultures of viable organisms containing recombinant DNA molecules shall be handled in a closed system (e.g., closed vessels used for the propagation and growth of cultures) or other primary containment equipment (e.g., Class III biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to prevent the escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system provided all physical containment requirements specified in Appendix G–II–C of the Guidelines are met.

Appendix K–IV–B. Culture fluids (except as allowed in Appendix K–IV–C) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant DNA molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant DNA molecules.

Appendix K–IV–C. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of culture fluids from one closed system to another shall be done in a manner which prevents the release of aerosols or contamination of exposed surfaces.

Appendix K–IV–D. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to HEPA filters or by other equivalent procedures (e.g., incineration) to prevent the release of viable organisms containing recombinant DNA molecules to the environment.

Appendix K–IV–E. A closed system or other primary containment equipment that has contained viable organisms containing recombinant DNA molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure. A validated sterilization procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant DNA molecules.

Appendix K–IV–F. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be operated so that the space above the culture level will be maintained at a pressure as low as possible, consistent with equipment design, in order to maintain the integrity of containment features.

Appendix K–IV–G. Rotating seals and other mechanical devices directly associated with a closed system used to contain viable organisms containing recombinant DNA molecules shall be designed to prevent leakage or shall be fully enclosed in ventilated housings that are exhausted through filters which have efficiencies equivalent to HEPA filters or through other equivalent treatment devices.

Appendix K–IV–H. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules and other primary containment equipment used to contain operations involving viable organisms containing recombinant DNA molecules shall include monitoring or sensing devices that monitor the integrity of containment during operations.

Appendix K–IV–I. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be tested for integrity of the containment features using the organisms that will serve as the host for propagating the recombinant DNA molecules. Testing shall be accomplished prior to the

introduction of viable organisms containing recombinant DNA molecules and following modification or replacement of essential containment features. Procedures and methods used in the testing shall be appropriate for the equipment design and for recovery and demonstration of the test organism. Records of tests and results shall be maintained on file.

Appendix K–IV–J. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be permanently identified. This identification shall be used in all records reflecting testing, operation, and maintenance and in all documentation relating to the use of this equipment for research production activities involving viable organisms containing recombinant DNA molecules.

Appendix K–IV–K. The universal biohazard sign shall be posted on each closed system and primary containment equipment when used to contain viable organisms containing recombinant DNA molecules.

Appendix K–IV–L. Emergency plans required by Section IV–B–3–f shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K–IV–M. Closed systems and other primary containment equipment used in handling cultures of viable organisms containing recombinant DNA molecules shall be located within a controlled area which meets the following requirments:

Appendix K–IV–M–1. The controlled area shall have a separate entry area. The entry area shall be a double-doored space such as an air lock, anteroom, or change room that separates the controlled area from the balance of the facility.

Appendix K–IV–M–2. The surfaces of walls, ceilings, and floors in the controlled area shall be such as to permit ready cleaning and decontamination.

Appendix K–IV–M–3. Penetrations into the controlled area shall be sealed to permit liquid or vapor phase space decontamination.

Appendix K–IV–M–4. All utilities and service or process piping and wiring entering the controlled area shall be protected against contamination.

Appendix K–IV–M–5. Hand-washing facilities equipped with foot, elbow, or automatically operated valves shall be located at each major work area and near each primary exit.

Appendix K–IV–M–6. A shower facility shall be provided. This facility shall be located in close proximity to the controlled area.

Appendix K–IV–M–7. The controlled area shall be designed to preclude release of culture fluids outside the controlled area in the event of an accidental spill or release from the closed systems or other primary containment equipment.

Appendix K–IV–M–8. The controlled area shall have a ventilation system that is capable of controlling air movement. The movement of air shall be from areas of lower contamination potential to areas of higher contamination potential. If the ventilation system provides positive pressure supply air, the system shall operate in a manner that prevents the reversal of the direction of air movement or shall be equipped with an alarm that would be actuated in the event that reversal in the direction of air movement were to occur. The exhaust air from the controlled area shall not be recirculated to other areas of the facility. The exhaust air from the controlled area may be discharged to the outdoors without filtration or other means for effectively reducing an accidental aerosol burden provided that it can be dispersed clear or occupied buildings and air intakes.

Appendix K–IV–N. The following personnel and operational practices shall be required:

Appendix K–IV–N–1. Personnel entry into the controlled area shall be through the entry area specifed in Appendix K–IV–M–I.

Appendix K–IV–N–2. Persons entering the controlled area shall exchange or cover their personal clothing with work garments such as jumpsuits, laboratory coats, pants and shirts, head cover, and shoes or shoe covers. On exit from the controlled area the work clothing may be stored in a locker separate from that used for personal clothing or discarded for laundering. Clothing shall be decontaminated before laundering.

Appendix K–IV–N–3. Entry into the controlled area during periods when work is in progress shall be restricted to those persons required to meet program or support needs. Prior to entry all persons shall be informed of the operating practices, emergency procedures, and the nature of the work conducted.

Appendix K–IV–N–4. Persons under 18 years of age shall not be permitted to enter the controlled area.

Appendix K–IV–N–5. The universal biohazard sign shall be posted on entry doors to the controlled area and all internal doors when any work involving the organism is in progress. This includes periods when decontamination procedures are in progress. The sign posted on the entry doors to the controlled area shall include a statement of agents in use and personnel authorized to enter the controlled area.

Appendix K–IV–N–6. The controlled area shall be kept neat and clean.

Appendix K–IV–N–7. Eating, drinking, smoking, and storage of food are prohibited in the controlled area.

Appendix K–IV–N–8. Animals and plants shall be excluded from the controlled area.

Appendix K–IV–N–9. An effective insect and rodent control program shall be maintained.

Appendix K–IV–N–10. Access doors to the controlled area shall be kept closed, except as necessary for access, while work is in progress. Serve doors leading directly outdoors shall be sealed and locked while work is in progress.

Appendix K–IV–N–11. Persons shall wash their hands when leaving the controlled area.

Appendix K–IV–N–12. Persons working in the controlled area shall be trained in emergency procedures.

Appendix K–IV–N–13. Equipment and materials required for the management of accidents involving viable organisms containing recombinant DNA molecules shall be available in the controlled area.

Appendix K–IV–N–14. The controlled area shall be decontaminated in accordance with established procedures following spills or other accidental release of viable organisms containing recombinant DNA molecules.

Appendix L—Release Into the Environment of Certain Plants

Appendix L–I—General Information

Appendix L specifies conditions under which certain plants as specified below, may be approved for release into the environment. Experiments in this category cannot be initiated without submission of relevant information on the proposed experiment to NIH, review by the RAC Plant Working Group, and specific approval by NIH. Such experiments also require the approval of the IBC before initiation. Information on specific experiments which have been approved will be available in ORDA and will be listed in Appendix L–III when the Guidelines are republished.

Experiments which do not meet the specifications of Appendix L–II fall under Section III–A and require RAC review and NIH and IBC approval before initiation.

Appendix L–II—Criteria Allowing Review by the RAC Plant Working Group Without the Requirement for Full RAC Review

Approval may be granted by ORDA in consultation with the Plant Working

Group without the requirement for full RAC review (IBC review is also necessary) for growing plants containing recombinant DNA in the field under the following conditions:

Appendix L–II–A. The plant species is a cultivated crop of a genus that has no species known to be a noxious weed.

Appendix L–II–B. The introduced DNA consists of well-characterized genes containing no sequences harmful to humans, animals, or plants.

Appendix L–II–C. The vector consists of DNA: (i) From exempt host-vector systems (Appendix C); (ii) from plants of the same or closely related species; (iii) from nonpathogenic prokaryotes or nonpathogenic lower eukaryotic plants; (iv) from plant pathogens only if sequences resulting in production of disease symptoms have been deleted; or (v) chimeric vectors constructed from sequences defined in (i) to (iv) above. The DNA may be introduced by any suitable method. If sequences resulting in production of disease symptoms are retained for purposes of introducing the DNA into the plant, greenhouse-grown plants must be shown to be free of such sequences before such plants, derivatives, or seed from them can be used in field tests.

Appendix L–II–D. Plants are grown in controlled access fields under specified conditions appropriate for the plant under study and the geographical location. Such conditions should include provisions for using good cultural and pest control practices, for physical isolation from plants of the same species outside of the experimental plot in accordance with pollination characteristics of the species, and for further preventing plants containing recombinant DNA from becoming established in the environment. Review by the IBC should include an appraisal by scientists knowledgeable of the crop, its production practices, and the local geographical conditions. Procedures for assessing alterations in and the spread of organisms containing recombinant DNA must be developed. The results of the outlined tests must be submitted for review by the IBC. Copies must also be submitted to the Plant Working Group of the RAC.

Appendix L–III—Specific Approvals

As of publication of the revised Guidelines, no specific proposals have been approved. An updated list may be obtained from the Office of Recombinant DNA Activities, National Institutes of Health, Building 31, Room 3B10, Bethesda, Maryland 20892.

(OMB's "Mandatory Information Requirements for Federal Assistance Program Announcements" (45 FR 39592) requires a statement concerning the official government programs contained in the *Catalog of Federal Domestic Assistance*. Normally NIH lists in its announcements the number and title of affected individual programs for the guidance of the public. Because the guidance in this notice covers not only virtually every NIH program but also essentially every Federal research program in which DNA recombinant molecule techniques could be used, it has been determined to be not cost effective or in the public interest to attempt to list these programs. Such a list would likely require several additional pages. In addition, NIH could not be certain that every federal program would be included as many Federal agencies, as well as private organizations, both national and international, have elected to follow the NIH Guidelines. In lieu of the individual program listing, NIH invites readers to direct questions to the information address above about whether individual programs listed in the *Catalog of Federal Domestic Assistance* are affected.)

Dated: April 18, 1986.

Thomas E. Malone,

Acting Director, National Institutes of Health.

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European Food Safety Authority

The EFSA Journal (2007) 587, 1-16

Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA[1]

Opinion of the Scientific Committee

(Question No EFSA-Q-2005-293)

Adopted on 19 November 2007

SCIENTIFIC COMMITTEE MEMBERS

Sue Barlow, Andrew Chesson, John D. Collins, Erik Dybing, Albert Flynn, Claudia Fruijtier-Pölloth, Anthony Hardy, Ada Knaap, Harry Kuiper, Pierre Le Neindre, Jan Schans, Josef Schlatter, Vittorio Silano, Staffan Skerfving, Philippe Vannier.

SUMMARY

A wide variety of microbial species are used in food and feed production. Some have a long history of apparent safe use, while others are less well understood and their use may represent a risk for consumers. Experience has shown that there is a need for a tool for setting priorities within the risk assessment of those microorganisms used in food/feed production referred to EFSA and consequently the subject of a formal assessment of safety. To meet this need a system was proposed for a pre-market safety assessment of selected groups of microorganisms leading to a "Qualified Presumption of Safety (QPS)". In essence this proposed that a safety assessment of a defined taxonomic group (*e.g.* genus or group of related species) could be made based on four pillars (establishing identity, body of knowledge, possible pathogenicity and end use). If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded (the qualification) the grouping could be granted QPS status. Thereafter, any strain of microorganism the identity of which could be unambiguously established and assigned to a QPS group would be freed from the need for further safety assessment other than satisfying any qualifications specified. Microorganisms not considered suitable for QPS would remain subject to a full safety assessment.

EFSA asked its Scientific Committee to consider whether this system could be used to harmonise approaches to the safety assessment of microorganisms across the various EFSA scientific panels. If so, the Committee was requested to develop a strategy for the introduction of an assessment system based on the QPS concept.

[1] For citation purposes: Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *The EFSA Journal* (2007) 587, 1-16

The Scientific Committee reviewed the range and numbers of microorganisms likely to be the subject of an EFSA opinion. They found that approximately 100 species of microorganisms have been or are expected to be referred to EFSA for a safety assessment; the majority being the result of notifications for market authorisation as sources of food and feed additives, food enzymes and plant protection products. A large majority of these species were found to fall within four broad groupings: i) Gram-positive non-sporulating bacteria; ii) *Bacillus* species, iii) yeasts and iv) filamentous fungi. Accordingly, bacteria, yeasts and fungi falling within these four groups were selected for an initial assessment of their suitability for QPS status. The Scientific Committee concluded that the weight of evidence available for many species falling within the first three of the four groups was sufficient to ensure that QPS status provided at least the same degree of confidence as a case-by-case safety assessment. However, the Committee found that, in the case of the filamentous fungi, the body of knowledge, particularly that relating to a history of use, was for a specific purpose and did not allow extrapolation to other uses to be made with confidence and so could not recommend QPS status for such fungi.

As the number of organisms considered suitable for QPS status is sufficiently extensive to cover a majority of the safety assessments involving microorganisms required of EFSA, the Scientific Committee concluded that the introduction of a QPS system for microorganisms would meet the objectives of providing a practical tool for setting priorities and avoiding the extensive investigations of organisms known not to cause concern. Although QPS status of metabolic products of microorganisms cannot be inferred from the QPS status of the production strain, the Committee considered that the system still had value for the assessment of strains used in the production of such products. Further work, however, would be required to extend the system to encompass those microorganisms used for biological control purposes.

Finally, in reaching its conclusion on the value of QPS as an assessment tool, the Scientific Committee recognised that there would have to be continuing provision for reviewing and modifying the list of organisms given QPS status. They recommended that the EFSA via its Science Directorate should take prime responsibility for this and should review the suitability for QPS status of the existing list and any additions at least annually. Reviews may occur more frequently as necessary but there should be a formal requirement that even when no changes are proposed, a statement should be made annually that QPS status is being maintained for the published list.

Key words:

Safety assessment, microorganisms, qualified presumption of safety, QPS, *Bacillus,* yeast, filamentous fungi, Gram-positive non-sporulating bacteria, lactic acid bacteria

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BACKGROUND

A wide variety of bacterial and fungal species are used in food and feed production, either directly or as a source of additives or food enzymes. Some of these have a long history of apparent safe use, while others are less well understood and may represent a risk for consumers. Experience has shown that there is a need for a tool for setting priorities within the risk assessment of those microorganisms used in the production of food/feed which are captured by present legislation and consequently the subject of a formal assessment of safety. Ideally such an assessment tool would allow the identification of risk without committing resources to extensive investigations of organisms known to be safe.

In 2002/3 a working group consisting of members of the former Scientific Committees on Animal Nutrition, Food and Plants of the European Commission proposed the introduction for selected microorganisms of a Qualified Presumption of Safety (QPS)[2].

In essence this proposed that a safety assessment of a defined taxonomic group (e.g. genus or group of related species) could be made independently of any particular pre-market authorisation process. If the taxonomic group did not raise safety concerns or, if safety concerns existed but could be defined and excluded (the qualification) the grouping could be granted QPS status. Thereafter any strain of microorganism the identity of which could be unambiguously established and assigned to a QPS group would be freed from the need for further safety assessment other than satisfying any qualifications specified. Those strains failing to satisfy a qualification would be considered hazardous and, in the absence of mitigating circumstances, unfit for purpose. Microorganisms not considered suitable for QPS would remain subject to a full safety assessment.

In April 2003, responsibility for the safety assessments of food/feed undertaken by the Scientific Committees of the Commission formally passed to the European Food Safety Authority (EFSA). Shortly after EFSA asked its own Scientific Committee to consider whether the approach to safety assessment of microorganisms proposed in the QPS document could be used to harmonise approaches to the safety assessment of microorganisms across the various EFSA scientific panels. In doing so, the Committee was requested to take into account the response of the stakeholders to the QPS approach. Their views had been sought by the three Commission Scientific Committees in 2002/3 and, subsequently, by EFSA at a Scientific Colloquium organised at the end of 2004 (EFSA 2005b).

The Scientific Committee concluded that QPS as a concept could provide a generic assessment system *for use within EFSA* that could be applied to all requests received for the safety assessments of microorganisms deliberately introduced into the food chain (EFSA 2005a). The benefits of the introduction of QPS would be a more transparent and consistent approach across the EFSA panels and the potential to make better use of resources by focussing on those organisms which presented the greatest risks or uncertainties.

[2] See http://ec.europa.eu/food/fs/sc/scf/out178_en.pdf

However, the Committee stressed that the body of knowledge about the organisms for which QPS is sought must be sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and predictable. Judgement as to whether the existing data are sufficient needed, in the view of the Committee, to be determined by an expert group established for this purpose and should be based on a weight-of-evidence approach.

On the basis of these conclusions the Scientific Committee recommended that EFSA should develop a strategy for the introduction of an assessment system based on the QPS concept. This should be limited to microorganisms introduced into the food chain or used as producer strains for food/feed additives until the robustness and value of such a system could be tested in practice.

EFSA accepted the recommendation of its Scientific Committee and proposed that the Committee should continue its assessment of the QPS system with a view to implementation[3]. Specifically, the Scientific Committee was asked first to establish which were the microorganisms most commonly referred to EFSA, including those used as a source of microbial products. Then, on the basis of this survey, to select relevant groups of microorganisms, examine the available data on safety and propose whether QPS status would be appropriate. If this proved possible in a significant number of cases then the Scientific Committee should consider how implementation of QPS across the various panels could be achieved.

TERMS OF REFERENCE AS PROVIDED BY EFSA

In response to its Opinion on the potential value of the QPS approach, the Scientific Committee is now requested by the European Food Safety Authority:

1. To establish which are the microorganisms most commonly referred to EFSA. This to include both organisms deliberately introduced into the food chain and those used as a source of microbial products entering the chain.

2. To select appropriate and relevant groups of microorganisms and, with the help of additional experts as necessary, to determine whether QPS status should be given.

3. Thereafter, to advise whether QPS represents a practical and robust method of safety assessment for microorganisms and, if so, to consider how the QPS could be applied across EFSA within the framework of the current and proposed legislation.

[3] See http://www.efsa.europa.eu/en/science/sc_commitee/sc_documents/1368.html

ACKNOWLEDGEMENTS

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ASSESSMENT

1. Questions to EFSA involving microorganisms

Approximately 100 species of microorganisms have been or are expected to be referred to EFSA for a safety assessment. The majority are the result of notifications for market authorisation as sources of food and feed additives, food enzymes and plant protection products. Others are the subject of the GMO and novel food/feed legislation. A few microbial species are also the subject of requests for opinions relating to consumer or animal safety not directly linked to product authorisation or to legislative requirements. Generally such requests relate to human enteropathogens or veterinary pathogens and so are beyond the scope of any consideration for QPS. Microorganisms referred to EFSA include both live organisms deliberately introduced into the food chain and those used as a source of food/feed additives and food enzymes. Individual species may be the subject of a single notification but more usually are found in several notifications. A large majority of these approximately 100 species were found to fall within four broad groupings:

1. Gram-positive non-sporulating bacteria (GPNS)
2. *Bacillus* species
3. Yeasts
4. Filamentous fungi

Accordingly, bacteria and fungi falling within these four groups were selected for an initial assessment of their suitability for QPS status. Organisms falling outside the four broad groups are infrequently notified. The Scientific Committee considers that such organisms could be considered for QPS at a later date but, in the interim, should continue to be assessed on a case-by-case basis. This would include viruses which are the occasional subject of notifications.

It should be noted that QPS status is taken to apply strictly to the microorganism and not to any traded product containing the organism or to a product of the microorganism. The Scientific Committee recognises that the final formulation may, on rare occasions, introduce additional hazards needing assessment. In addition, QPS status informs only on the safety of a microorganism and should be used without prejudice to any other requirements of legislation.

2. Consideration for QPS status

The suitability of various taxonomic groups falling under the four broad headings for QPS status was examined by working groups of the Scientific Committee. Their preliminary proposals for suitable candidates for QPS status and the documentation supporting these conclusions were made available for public consultation. Interested parties were invited in particular to comment on whether the weight of evidence presented was sufficient to ensure that QPS status provides at least the same degree of confidence as a case-by-case safety assessment, whether this was adequately documented and whether there were issues that have not been sufficiently considered.

Responses from some thirty individuals and organisations were received, principally from trade organisations and companies, but including scientists from a number of European academic institutions, trade associations and national food safety authorities. Relatively few comments were directed to the specifics of the analyses of suitability for QPS status. Most were concerned with more general issues, some positive and others raising matters which were considered insufficiently developed. Concerns remain about the status of QPS in relation to existing and future legislation recognising that the application of QPS by EFSA could have implications for risk managers. The issue of how a QPS list would be maintained was also raised, since scientific developments might require a species to be withdrawn or allow a species to be added. There were also concerns about the continuing emphasis on the absence of acquired antibiotic resistance determinants as a qualification in bacteria and that a restriction on end-use was not more generally applied to allow additional organisms to be considered suitable for QPS, particularly amongst the filamentous fungi.

All of the comments received were taken into consideration when reviewing and revising the conclusions on suitability for QPS status. The organisms which the Scientific Committee considers suitable for QPS status is given in Table 1. For convenience this is given as a list of presently-recognised species. Where QPS status is proposed, the Scientific Committee is satisfied that the body of knowledge available is sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and capable of exclusion.

A summary of some of the specific issues arising within each of the four broad groupings is highlighted below. Otherwise the scientific justifications for inclusion in this list are given in the individual reports on the four groupings (Appendices A-D). These reports take a common structure based around the four pillars of the assessment for suitability for QPS status (establishing identity, body of knowledge, possible pathogenicity and end use) following the general scheme previously published[4]. The current state of knowledge and the very different nature of the organisms involved, however, have meant that the emphasis and content within the four reports inevitably differs. Each individual report is a summary of extended considerations based on a thorough review of the available scientific literature and the knowledge and experience of the scientists involved. Where literature is cited, this is to support key conclusions or more generally to illustrate an issue.

[4]See http://www.efsa.europa.eu/etc/medialib/efsa/science/colloquium_series/no2_qps/948.Par.0015.File.dat/summary_report1.pdf , page 16

Table 1. **List of taxonomic units proposed for QPS status**

Gram-Positive Non-Sporulating Bacteria[5]			
Species			**Qualifications**
Bifidobacterium adolescentis *Bifidobacterium animalis*	*Bifidobacterium bifidum* *Bifidobacterium breve*	*Bifidobacterium longum*	
Corynebacterium glutamicum			QPS status applies only when the species is used for production purposes.
Lactobacillus acidophilus *Lactobacillus amylolyticus* *Lactobacillus amylovorus* *Lactobacillus alimentarius* *Lactobacillus aviaries* *Lactobacillus brevis* *Lactobacillus buchneri* *Lactobacillus casei* *Lactobacillus crispatus* *Lactobacillus curvatus* *Lactobacillus delbrueckii*	*Lactobacillus farciminis* *Lactobacillus fermentum* *Lactobacillus gallinarum* *Lactobacillus gasseri* *Lactobacillus helveticus* *Lactobacillus hilgardii* *Lactobacillus johnsonii* *Lactobacillus kefiranofaciens* *Lactobacillus kefiri* *Lactobacillus mucosae* *Lactobacillus panis*	*Lactobacillus paracasei* *Lactobacillus paraplantarum* *Lactobacillus pentosus* *Lactobacillus plantarum* *Lactobacillus pontis* *Lactobacillus reuteri* *Lactobacillus rhamnosus* *Lactobacillus sakei* *Lactobacillus salivarius* *Lactobacillus sanfranciscensis* *Lactobacillus zeae*	
Lactococcus lactis			
Leuconostoc citreum	*Leuconostoc lactis*	*Leuconostoc mesenteroides*	
Pediococcus acidilactici	*Pediococcus dextrinicus*	*Pediococcus pentosaceus*	
Propionibacterium. freudenreichii			
Streptococcus thermophilus			

[5] Absence of acquired antibiotic resistance should be systematically demonstrated unless cells are not present in the final product (EFSA, 2005c).

Table 1 (cont'd). **List of taxonomic units proposed for QPS status**

Bacillus[6]			
Species			**Qualifications**
Bacillus amyloliquefaciens *Bacillus atrophaeus* *Bacillus clausii* *Bacillus coagulans* *Bacillus fusiformis*	*Bacillus lentus* *Bacillus licheniformis* *Bacillus megaterium* *Bacillus mojavensis*	*Bacillus pumilus* *Bacillus subtilis* *Bacillus vallismortis* *Geobacillus stearothermophillus*	Absence of emetic food poisoning toxins with surfactant activity.* Absence of enterotoxic activity.*

* When strains of these QPS units are to be used as seed coating agents, testing for toxic activity is not necessary, provided that the risk of transfer to the edible part of the crop at harvest is very low (section 4.3 of Appendix B).

Yeasts			
Species			**Qualifications**
Debaryomyces hansenii			
Hanseniaspora uvarum			
Kluyveromyces lactis	*Kluyveromyces marxianus*		
Pichia angusta	*Pichia anomala*		
Saccharomyces bayanus	*Saccharomyces cerevisiae*	*Saccharomyces pastorianus (synonym of Saccharomyces carlsbergensis)*	S. cerevisiae, subtype S. boulardii is contraindicated for patients of fragile health, as well as for patients with a central venous catheter in place. A specific protocol concerning the use of probiotics should be formulated
Schizosaccharomyces pombe			
Xanthophyllomyces dendrorhous			

2.1. Gram-positive non-sporulating bacteria

Many of the referred microorganisms falling within this grouping are normal inhabitants of the digestive tract of humans and livestock or are commonly used in the preparation of foods and feed. Consequently, there has been a long history of human exposure with only very occasional reports of adverse effects and then only amongst compromised individuals. However, amongst the microorganisms referred to EFSA, two particular groups of microorganisms raised issues requiring particular attention. The most important was the consideration given to the enteroccoci. Bacteria in the genus *Enterococcus* are amongst the leading causes of community- and hospital-acquired (nosocomial) infections. Infections often result from *Enterococcus faecalis*, but there are also virulent strains found within *E. faecium,* the species of *Enterococcus* most commonly deliberately introduced into the food chain. Although a considerable amount is known about the virulence determinants in enteroccoci, given the prevalence of enteroccocal infections, the Scientific Committee verged on the side of caution and did not propose QPS status for *Enterococcus* species, as at present it is not possible readily to distinguish between virulent and non-virulent strains without resorting to the level of investigation used in a case-by-case assessment. This position could be reviewed as it becomes clearer which are the key determinants of virulence and as suitable molecular probes for such determinates are developed.

The second issue highlighted the debate about the distinction between opportunistic infections, of which almost all microorganisms that humans commonly encounter are capable, and pathogenicity. Many *Lactobacillus* species have been occasionally encountered in clinical specimens, the clinical significance of which is not always clear. Such occurrences have almost invariably been associated with immunocompromised patients, those who had suffered surgical or accidental insult or who had a serious underlying illness, and remain rare. As such, these infections can be considered opportunistic and beyond the capacity of any safety assessment to exclude. Although a number of *Lactobacillus* spp. have been reported to infect otherwise healthy individuals with a history of rheumatic endocarditis or following heart valve replacement, one species, *L. rhamnosus,* appears to predominate. This organism could be considered on the edge of being defined as pathogenic. The Scientific Committee took the view that the at-risk population is not placed at added risk by the use of *L. rhamnosus* in food/feed and so confirmed the proposed QPS status of this bacterium. The Committee considers that this is a decision which should be reviewed at regular intervals.

2.2. Bacillus species

The Scientific Committee is of the opinion that the use of strains from the *B. cereus* group should be avoided whenever there is a possibility of human exposure whether intended or incidental. The *B. cereus* group is therefore excluded from consideration for QPS status.

There is an artificial distinction held between *B. cereus* and *B. thuringiensis* (used for plant protection) which has little scientific basis. The plasmid encoding the insecticidal enterotoxin, which provides the phenotypic distinction for *B. thuringiensis,* is readily lost, particularly when grown at 37°C, leaving an organism indistinguishable from *B. cereus*. Consequently it is likely

that *B. thuringiensis* has been the causative organism of some instances of food poisoning but identified as *B. cereus* because clinical investigations would have failed to recognise the distinguishing features characteristic of *B. thuringiensis*.

However, the Scientific Committee recognises that *B. thuringiensis* has value to the industry as a means of biological pest control and that its widespread use for this purpose may not lead to significant human exposure.

Although occasional strains of *Bacillus* not falling within the *B. cereus* group also produce human enterotoxins, experience gained with *B. cereus* has provided the tools for their exclusion. This is recognised as a qualification in recommending QPS status for other *Bacillus* species.

2.3. Yeasts

Yeasts used in food production, particularly brewers/bakers yeast, are considered amongst the safest of microorganisms. However, even amongst this group there are reports of very occasional invasive infections. A sub-type of *Saccharomyces cerevisiae*, commonly referred to as *Saccharomyces boulardii* has been used as an adjunct to the antibiotic treatment of persistent diarrhoea often arising from *Clostridium difficile* infections, to reduce the likelihood of reoccurrence. This subtype has been isolated from the blood in approximately half of the reported invasive infections involving *Saccharomyces*. The majority of these cases occurred in compromised individuals. However this has to be placed in context. Despite the continuous and universal exposure to this yeast there have been less than a hundred documented cases of invasive infection by *Saccharomyces* spp., half of which occurred amongst those undergoing aggressive antibiotic treatment. As the at-risk group results from a strictly medical application without implication for the healthy population, the Scientific Committee did not see a reason to exclude *Saccharomyces* and so confirmed the proposed QPS status of this genus.

2.4. Filamentous fungi

The filamentous fungi could not be included within the QPS system. Although in many cases there has been a history of use, this has been for specific purposes such as the production of citric acid or processing enzymes. The body of knowledge that has developed has, in consequence, centred on these uses. However, many of the filamentous fungi used for production purposes are known to produce substances of potential concern (mycotoxins, *etc*).

The strength of the QPS lies in the ability to provide a generic system of safety assessment. This can be extended in scope by introducing a limited number of qualifications, allowing the majority of a taxonomic group to be assumed safe while excluding a minority of problematic strains. On examination, while it was possible to identify specific metabolites of filamentous fungi which should be excluded, it was not possible to be sure that these represented the totality of substances of concern capable of being produced by the taxonomic unit. Introducing restricted use as a qualification did not offer a solution since purpose does not offer any reassurance on overall metabolic capacity. The absence of undesirable compounds in one or

more selected production strains does not allow extrapolation to all strains within the selected taxonomic unit.

3. QPS as a tool for the assessment of the safety of microorganisms

3.1. Value to EFSA of QPS as an assessment tool

The list of organisms in Table 1 is sufficiently extensive to cover a large majority of the safety assessments involving microorganisms required of EFSA. Consequently, the Scientific Committee considers the introduction of a QPS system for microorganisms would meet the original objectives of providing a practical tool for setting priorities and avoiding the extensive investigations of organisms already known to be safe.

Although QPS status of metabolic products of microorganisms cannot be inferred from the QPS status of the production strain, the system still has considerable value for the assessment. Microbial products (*e.g.* enzymes, organic acids, amino acids) used in food/feed are rarely the primary source of possible concerns. Most case-by-case assessments focus on the presence of other metabolites which might be carried through to the final product. The QPS status of the production strain would provide the assurance that any metabolites other than that intended which are found in the final product would not be hazardous. This would simplify and greatly assist the assessment process.

Genetically modified microorganisms (GMMs) are the subject of specific legislation, whether used directly and released into the environment (Regulation 1829/2003[6], Directive 2001/18/EC[7]) or used under containment as a source of specific products (Directive 98/81/EC[8]). In the simplest case, that of self-cloning, the QPS status of the parent strain should be accepted and extrapolated to the modified strain without further need for assessment (EFSA 2006). Whenever foreign genetic material is introduced in a GMM, QPS is most likely to be of relevance to the recipient strain and only rarely to the source of the introduced trait. When the recipient strain has QPS status, then the assessment is free to focus on the introduced trait(s), relying on the reviewed body of knowledge to exclude potential hazards arising from the recipient strain.

QPS in its present form does not offer a generic approach to the safety assessment of microorganisms used as biological control agents. Most are based on filamentous fungi or bacteria hazardous to humans when they are directly exposed. Indeed the protection offered to plants by such organisms may depend, in part at least, on these toxic principles. However, the Scientific Committee considers that it may be possible to devise robust use qualifications which would allow a QPS approach in the future. Such qualifications would have to include a consideration of effects on non-target species.

[6] See http://eur-lex.europa.eu/LexUriServ/site/en/oj/2003/l_268/l_26820031018en00010023.pdf
[7] See http://eur-lex.europa.eu/LexUriServ/site/en/oj/2001/l_106/l_10620010417en00010038.pdf
[8] See http://eur-lex.europa.eu/LexUriServ/site/en/oj/1998/l_330/l_33019981205en00130031.pdf

3.2. Exclusion from the QPS list

Only a positive list of microorganisms judged suitable for QPS status is given in Table 1. Exclusion from this list was for a variety of reasons. Many microorganisms commonly encountered in food production were not considered because they are not presently the subject of pre-market authorisations and so would not be notified to EFSA. Other microorganisms were considered (*e.g.* the enterococci), but the potential risks associated with their use could not be fully defined or, where recognised, the tools necessary for the exclusion of hazardous strains were considered insufficient. Often the body of knowledge, particularly that relating to a history of use, was for a specific purpose and did not allow extrapolation to other uses to be made with confidence.

It should be stressed that the absence of a particular organism from the list of microorganisms judged suitable for QPS does not necessarily imply any risk associated with its use. Individual strains may be safe but this cannot be judged from the existing knowledge of the taxonomic unit to which it belongs. Consequently, all microorganisms not considered for QPS or not considered as suitable would remain subject to a full safety assessment.

3.3. Maintenance of QPS list

In reaching its conclusion on the value of QPS as an assessment tool, the Scientific Committee recognises that there would have to be continuing provision for reviewing and modifying the list of organism given QPS status. EFSA must be able to respond to any new information such as epidemiological data which might suggest that an inclusion on the list should be reconsidered. Similarly, there must be provision for additions. It is likely that the rapid developments in microbial genomics, the full annotation of genomes and the ability to predict metabolic pathways accurately from such annotation will allow inclusion of organisms not presently listed. Proposals for additions could arise from a variety of source including actual notifications to EFSA or at the request of interested parties. However, whatever the route and source of information on which on a judgement of suitability for QPS status is made, the assessment itself and the final judgement on whether to include or exclude must remain within EFSA's purlieu.

The Scientific Committee is of the opinion that responsibility for the maintenance and development of the QPS system should be the responsibility of EFSA via its Science Directorate. This would ensure that the system has the continuing necessary support and that there would be a continuity of approach. The Scientific Committee suggests that the EFSA should review at least annually the received notifications involving microorganisms and the suitability for QPS status of any new additions. The review should also consider suggestions arising from elsewhere (*e.g.* industry, Member States) for possible additions to or deletions from the QPS list. Reviews may occur more frequently but there should be a formal requirement that even where no changes are proposed a statement should be made annually that QPS status is being maintained for the published list.

3.4. Implementation of QPS within EFSA

For QPS to be effective it must be implemented across EFSA for all safety considerations of microorganisms intentionally added to the food chain, regardless of purpose. There should be full harmonisation and implementation of QPS in all EFSA Panels wherever applicable.

For those panels concerned with risk assessments leading to market authorisations there will be an interim period after the introduction of the QPS system during which Dossiers will reflect previous requirements and contain data made unnecessary for those organisms listed in Table 1. The Scientific Committee suggests that this should be managed and a consistent response adopted in which QPS status is given priority but accompanied by an acknowledgement that data has been provided which is consistent with the QPS status of the organism(s). During this initial period it is suggested that the Scientific Committee should monitor the introduction of QPS and act as a forum in which any difficulties could be resolved.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

The Scientific Committee is of the view that the weight of evidence available for the bacterial and fungal species listed in Table 1 is sufficient to ensure that QPS status provides at least the same degree of confidence as a case-by-case safety assessment,

As the number of organisms considered suitable for QPS status is sufficiently extensive to cover a majority of the safety assessments involving microorganisms required of EFSA, the Scientific Committee considers the introduction of a QPS system for microorganisms would meet the objectives of providing a practical tool for setting priorities and avoiding the commitment of resources to extensive investigations of organisms known not to cause concern.

In reaching its conclusion on the value of QPS as an assessment tool, the Scientific Committee recognises that there would have to be continuing provision for reviewing and modifying the list of organism given QPS status.

RECOMMENDATIONS

The Scientific Committee recommends that a QPS system for microorganisms should be introduced initially covering the organisms listed in Table 1 and that should be implemented across EFSA for all safety considerations of microorganisms intentionally added to the food chain, regardless of purpose. Thereafter, and based on the experience gained from its use in practice, the extension of the QPS system to microbial products could be explored.

EFSA should take prime responsibility for the maintenance and development of QPS and should review at least annually the suitability for QPS status of the existing list and/or of any additions. Reviews may occur more frequently as necessary but there should be a formal requirement that even where no changes are proposed a statement should be made annually that QPS status is being maintained for the published list.

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APPENDIX A. Scientific report on the Assessment of Gram-Positive Non-Sporulating Bacteria

Many of the species included in this broad grouping are common constituents of the normal gut flora of humans and livestock although their occurance and numbers are host dependent. Additionally, species of Gram-positive bacteria constitute common components of the microbial community of food and, for their relevant role in food fermentation; these microorganisms have been deliberately introduced into food as starter cultures. In addition, several bacterial strains belonging to this group have a long history of apparent safe use as food starter cultures, feed additives (*e.g.* animal probiotics and silage inoculants) and source of additives (*e.g.* enzymes and amino acids). Based on their habitat and their extensive application in the food and feed sector, many species were judged potentially suitable for their safety to be assessed by the "Qualified Presumption of Safety" (QPS) methods, according to (EFSA 2005). The following genera, all belonging to the phylum *Firmicutes*, have been considered: *Bifidobacterium, Corynebacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Propionibacterium* and *Streptococcus.*

1 *Bifidobacterium*

Bifidobacteria are part of the normal gut microbiota of adults and are also one of the first genera to colonise the gut of infants. In addition, they are normal inhabitants of the gut of animals. A limited number of *Bifidobacterium* species have a history of use in dairy products, especially sour milk products like yoghurts and more recently yoghurt and fermented milk drinks.

1.1 Taxonomic unit defined

Bifidobacteria belong to the *Actinomycetes* branch of phylum Firmicutes. They are non-motile, non-sporeforming rods of variable appearance, usually curved and clubbed, and are often branched including Y and V forms. They are normally strictly anaerobic, although some species and strains tolerate oxygen. The type species is *Bifidobacterium bifidum.* Bifidobacteria are saccharolytic organisms and they have the ability to ferment glucose, galactose and fructose. Glucose is fermented via the fructose-6-phosphate shunt to acetic and lactic acid. Differences occur between species in their ability to ferment other carbohydrates and alcohols.

The genus consists currently of following species: *Bifidobacterium adolescentis, B. angulatum, B. animalis* subsp. *Animalis, B. animalis* subsp. *lactis, B. asteroides, B. bifidum, B. boum, B. breve, B. catenulatum, B. choerinum, B. coryneforme, B. cuniculi, B. dentium, B. gallicum, B. gallinarum, B. indicum, B. longum, B. magnum, B. merycicum, B. minimum, B. pseudocatenulatum, B. pseudolongum* subsp. *globosum, B. pseudolongum* subsp.

pseudolongum, *B. psychraerophilum*, *B. pullorum*, *B. ruminantium*, *B. saeculare*, *B. scardovii*, *B. subtile*, *B. thermacidophilum* subsp. *porcinum*, *B. thermacidophilum* subsp. *thermacidophilum*, *B. thermophilum* .

1.2 Is the body of knowledge sufficient?

The characteristics and habitat of the species of the genus *Bifidobacterium* are well known. The number of established or proposed species has increased only slightly during recent years.

Only a few species have a long history of use in industrial applications. Bifidobacteria are mainly exploited in dairy products like yogurts or yogurt drinks, but also a whole range of sour milk and other milk based products. Occasionally they are also used in feed in combination with other genera. In Europe only a few species are used (*B. animalis, B. longum, B. breve*, *B. bifidum* and *B. adolescentis,*) and often applied in combination with lactic acid bacteria (Reuter 1990; Reuter 1997; Klein, Pack *et al.* 1998; Reuter 2002).

The genome sequences of *B. longum* (Schell, Karmirantzou *et al.* 2002) and *B. breve* have been determined, while the genome sequencing project of *B. adolescentis* is ongoing.

1.3 Are there safety concerns?

Humans. Safety concerns are so far related mainly only to one species, *B. dentium*, which has been associated with dental caries. It has also been isolated from a case of peritonsillar abscess together with other anaerobes (Civen, Vaisanen *et al.* 1993) and, under its previous designation "*Actinomyces eriksonii*", from pulmonary and subcutaneous abscesses (Slack 1974). Occasionally, other species have been reported to be isolated from human clinical cases, but none of them was the primary cause of disease. Only immunocompromised hosts were infected (Crociani, Biavati *et al.* 1996). These species are not used as food or feed supplements. None of the bifidobacteria used for industrial purposes have been associated with human clinical disease.

Although there are few studies on the antibiotic resistance of bifidobacteria strains, the presence of the acquired tetracycline resistance gene *tet*(W) has been reported in *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum* (Kastner, Perreten et *al.* 2006; Masco, Van Hoorde *et al.* 2006).

Livestock. No report can be found on safety concerns related to *Bifidobacteria* in animals.

1.4 Can the safety concerns be excluded?

There are apparently no specific safety concerns regarding the genus *Bifidobacterium* (especially concerning *B. animalis; B. longum, B. breve, B. adolescentis,* and *B. bifidum*) with the exception of the species associated with dental caries, *B. dentium*. Susceptibility to antibiotics should be assessed as defined by the EFSA opinion (EFSA 2005) for each strain.

1.5 Units proposed for QPS status

Due to the long history of safe use of *B. adolescentis, B. animalis; B. longum, B. breve* and *B. bifidum*, these species are proposed for QPS status. Other species could be included subsequent to their industrial application with the exception of the species associated with dental caries (*B. dentium*).

2 *Corynebacterium*

Corynebacterium glutamicum is a soil bacterium widely used for the biotechnological production of amino acids. Amino acid producing strains have been selected and improved by mutagenesis as well as by using recombinant DNA technology. *C. glutamicum* belongs to a genus which also includes significant human pathogenic bacteria. Although some *Corynebacterium* species have been detected as components of the bacterial community of cheese surface, only *C. glutamicum* is considered of relevance for feed and food sectors. Only this species has been considered for the QPS assessment because of its significant role in the industrial production of amino acids.

2.1 Taxonomic unit defined

The genus *Corynebacterium* belongs to a branch of the *Actinomycetales* that also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus*. Bacterial species belonging to this branch of the Gram-positive bacteria share particular characteristics, such as high G+C content (47– 74%) and a specific cell envelope organisation, mainly characterised by the presence of peptidoglycan, arabinogalactan and mycolic acids. The genus currently contains 63 species, which colonise different environments.

2.2 Is the body of knowledge sufficient?

The characteristics, the physiology and the genetics of *C. glutamicum* are well known. The genome sequence of this industrial bacterium has been determined (Kalinowski, Bathe *et al.* 2003), reflecting the considerable biotechnological importance of these organisms.

2.3 Are there safety concerns?

Corynebacterium glutamicum plays an important role in the amino acid fermentation industry. No safety concerns are reported for this bacterial species for humans and animals, and no information on the presence of acquired antibiotic resistances in this bacterial species is available. However, it should be kept in mind that the direct exposure of consumers to this bacterial species is expected to be very low.

2.4 Can the safety concerns be excluded?

C. glutamicum has generally been considered to be non-pathogenic and no safety concerns are envisaged. However, its history of use is as a source of amino acids and has not, to date, involved the direct and deliberate exposure of humans or livestock.

2.5 Units proposed for QPS status

There is a long history of safe use of *C. glutamicum* as an amino acid producer; consequently, *C. glutamicum* is proposed for QPS status with the qualification that this status applies only when the species is used for production purposes only.

3 *Enterococcus*

Enterococci are significant strains found naturally in some foods and food products. They are often part of the natural microbiota involved in flavour and texture development resulting from fermentation, but can also occur as contaminants of foods. These organisms are used as starter cultures in food products, such as cheese, as probiotic cultures for humans and animals and as silage additives (Franz, Stiles *et al.* 2003; Foulquie Moreno, Sarantinopoulos *et al.* 2006). These organisms exist as normal human commensals but they are also associated with human infections. The *Enterococcus* genus is of particular medical relevance because of its increased incidence as a cause of disease in hospital-acquired (nosocomial) infections and because the available antibiotic therapies are being compromised by evolving transmissible antibiotic resistance. Enterococci harbour virulence factors on mobile genetic elements and they are noted for their capacity to exchange genetic information by bacterial mating. Gene transfer processes are well characterised in *Enterococcus* (Kozlowicz, Dworkin *et al.* 2006).

3.1 Taxonomic unit defined

In recent years, the genus *Enterococcus* has undergone considerable changes in taxonomy. Since the recognition of *Enterococcus* as a separate genus (Schleifer 1984), several new species have been described as a result of improvements in the methods for their identification, combined with a growing interest in their role as opportunistic pathogens. The genus consists currently of the following species: *E. aquimarinus, E. asini, E. avium, E. canintestini, E. canis, E. casseliflavus, E. cecorum, E. columbae, E. devriesei, E. dispar, E. durans, E. faecalis, E. faecium, E. gallinarum, E. gilvus, E. haemoperoxidus, E. hermanniensis, E. hirae, E. italicus, E. malodoratus, E. moraviensis, E. mundtii , E. pallens, E. phoeniculicola, E. pseudoavium, E. raffinosus, E. ratti, E. saccharolyticus, E. silesiacus, E. sulfureus* and *E. termitis.*

3.2 Is the body of knowledge sufficient?

The characteristics and habitat of most of enterococci species are well known. Strains of *E. faecium* have a long history of apparent safe use in industrial and agricultural applications. However, members of the same species and same genus are a major concern in clinical practice for their prevalence in nosocomial infections (Zirakzadeh and Patel 2006). The biology, physiology and the genetics of *E. faecalis* are well known. A number of virulence factors and antibiotic resistance determinants have been identified and characterised. Increased information on this genus is being derived from analysis of the sequence of *E. faecalis* (Paulsen, Banerjei *et al.* 2003) and *E. faecium* (http://genome.ornl.gov/microbial/efae/) genomes (Lepage, Brinster *et al.* 2006). Although the information on enterococci as infectious agents is available there is still a lack of knowledge on the role of the food chains as a source of virulent enterococci.

3.3 Are there safety concerns?

Humans. Enterococci are among the leading causes of both community and hospital-acquired infections. Different virulence factors, implicated in the pathogenesis of enterococci, have been described. In *E. faecalis* virulence determinants, such as citolysin operon, *esp,* and the gene encoding aggregation substance are clustered on a large pathogenicity island, a genetic element of approximately 150 kilobases in size (Shankar, Baghdayan *et al.* 2002; Shankar, Coburn *et al.* 2004). Cytolysin has been demonstrated to contribute to bacterial virulence (Coburn and Gilmore 2003). Surface adhesin coded on pheromone plasmids, was shown to be a virulence factor involved in the adhesion of enterococci to eukaryotic cell surfaces and in the production of experimental infections (Chandler, Hirt *et al.* 2005). Gelatinase is an additional virulence factor; *E. faecalis* mutants with an insertion disruption in the *gelE* or *fsr* operon, the gelatinase regulatory system, showed significant delays in mortality in a mouse peritonitis model (Singh, Nallapareddy *et al.* 2005). Moreover, it has been shown that in *E. faecalis* gelatinase is important for the translocation across human enterocyte-like T84 cells (Zeng, Teng *et al.* 2005). Virulence determinants have also been detected in *E. faecium.*

The safety concerns related to these bacteria are heightened by the antibiotic resistance of enterococci: these organisms show intrinsic resistance to a variety of antibiotics, such as aminoglycosides and β-lactams. Furthermore, enterococci have acquired genetic determinants for antibiotic resistances, and among them the resistance to high levels of glycopeptides is of major concern (Courvalin 2006).

Food strains of *Enterococcus* were found to harbour antibiotic resistance genes (Rizzotti, Simeoni *et al.* 2005; Hummel, Holzapfel *et al.* 2007), and high-frequency gene transfer of antibiotic resistance and virulence determinants in food within the enterococcal community in the absence of selective pressure was observed in food (Cocconcelli, Cattivelli *et al.* 2003). Occurrence of virulence factors in food enterococci is reported (Semedo, Almeida Santos *et al.* 2003), although

they have fewer virulence determinants than did clinical strains (Eaton and Gasson 2001; Mannu, Paba *et al.* 2003; Lepage, Brinster *et al.* 2006).

Livestock. Species of genus *Enterococcus,* such as *E. durans* and *E. hirae*, have been associated with infections in chickens (Chadfield, Christensen *et al.* 2005; Abe, Nakamura *et al.* 2006).

3.4 Can the safety concerns be excluded?

Some strains of *E. faecium* show a long history of apparent safe use in food or feed and lack many of the virulence determinants described to date. However, since there are significant safety issues regarding *Enterococcus* strains, and since the determinants of virulence are not fully understood, concerns cannot be excluded. Considering the number of infections linked to *Enterococcus,* a case-by-case approach should be adopted.

3.5 Units proposed for QPS status

Due to safety concerns and the lack of information on the safety, no members of the genus *Enterococcus* can be proposed for QPS status.

4 *Lactobacillus*

The genus *Lactobacillus* is a wide and heterogeneous taxonomic unit, comprising the rod-shaped lactic acid bacteria. This genus encompasses more than 100 different species with a large variety of phenotypic, biochemical and physiological properties. Many of the species are significant constituents of the normal gut flora of humans and livestock although their occurrence and numbers are host dependent. Several species of the genus are intentionally introduced in the food chains, being involved in a range of food and feed fermentations and applied as probiotics for humans and animals.

4.1 Taxonomic unit defined

As for other lactic acid bacteria, lactobacilli belong to the phylum *Firmicutes*. They are rod-shaped, non-motile and non-sporeformers. Classically, the *Lactobacillus* genus is divided into three groups: group 1, obligate homofermentative, group 2, facultative heterofermentrative and group 3 obligate heterofermentrative (for a review, see Axelsson 2004). The application of phylogenetic molecular taxonomy and 16S rRNA gene sequence analysis resulted in several changes within the taxonomy of this genus, with an increase in the number of species. At present 112 species belong to the genus *Lactobacillus*. Several molecular methods are available for the identification of lactobacilli to species level.

4.2 Is the body of knowledge sufficient?

The characteristics and habitat of most of *Lactobacillus* species are well known. Some of the species of this genus have a long history of apparent safe use in industrial and agricultural applications. Lactobacilli are used as starter cultures in a variety of food fermentation, such as dairy products, fermented and cured meats, fermented vegetables, sourdough and silage. Moreover, they are among the dominant populations in microbial communities of traditional fermented foods, being part of the natural starter cultures. Increased information on this genus is being derived from the sequence analysis of several genomes of *Lactobacillus* species.

4.3 Are there safety concerns?

Members of the *Lactobacillus* genus are daily consumed in large quantities in a variety of fermented foods by people of all ages, ethnic groups and health status with apparently no ill effects. Apart from their possible involvement in the development of dental caries, lactobacilli have generally been considered to be non-pathogenic. However, there has been an increasing number of reports that these organisms might occasionally be involved in human disease (Sharpe, Hill *et al.* 1973; Gasser 1994; Salminen, Rautelin *et al.* 2006). A variety of different *Lactobacillus* species has been recovered from human clinical specimens. These include *L. rhamnosus, L. fermentum, L. plantarum, L. casei, L. jensenii, L. salivarius, L. gasseri, L. salivarius,* and *L. acidophilus.* Clinical conditions from which these species were derived were chiefly subacute endocarditis and bacteremia or systemic septicemia, but also included abscesses, chorioamnionitis, and urosepsis (Lorenz, Appelbaum *et al.* 1982; Dickgiesser, Weiss *et al.* 1984; Salminen, Tynkkynen *et al.* 2002; Salminen, Rautelin *et al.* 2004; Salminen, Rautelin *et al.* 2006). Even the strain *L. rhamnosus* ATCC 53103, used as human probiotic, has occasionally been encountered in clinical specimens such as blood or pus samples (Rautio, Jousimies-Somer *et al.* 1999; Salminen, Tynkkynen *et al.* 2002; Salminen, Rautelin *et al.* 2004; De Groote, Frank *et al.* 2005; Salminen, Rautelin *et al.* 2006). However, Salminen and co-workers (Salminen, Rautelin *et al.* 2006) demonstrated that increased probiotic use of *L. rhamnosus ATCC* 53103 had not led to an increase in *Lactobacillus* bacteraemia. Furthermore, it has been demonstrated that strains isolated from clinical samples, show phenotypic, differences from probiotic *L. rhamnosus* strains (Klein, Hack *et al.* 1995; Ouwehand, Saxelin *et al.* 2004). Many of the patients with apparent *Lactobacillus* infection were immunocompromised or had other severe underlying illnesses. As far as endocarditis due to lactobacilli is concerned, this infection usually develops on the basis of preceding anatomical alterations of the heart valves. There are indications, however, that good adhesion properties of lactobacilli and, thus, of probiotic strains, might be a potential risk for bacteremia (Apostolou, Kirjavainen *et al.* 2001). In conclusion, most of the *Lactobacillus* species described to date can rightly be considered to be non-pathogenic to humans (Bernardeau, Guguen *et al.* 2006). Only certain strains of *L. rhamnosus* may be considered to be potential human opportunistic pathogens because they not only affect severely immunocompromised, but also

immunologically healthy individuals with a history of rheumatic endocarditis or heart valve replacement.

Several examples of antibiotic resistant lactobacilli isolated from food or from the gut of animals exist. Acquired genes for antibiotic resistance have been detected in *Lactobacillus* species: *tet*(M) has been found in *L. plantarum, L. brevis, L. sakei* and *L. curvatus* (Danielsen 2002; Gevers, Danielsen *et al.* 2003) and *tet*(S) in *L. plantarum* (Huys, D'Haene *et al.* 2006). Erythromycin resistance determinants *erm*(B) has been found in *L. plantarum, L. salivarius, L. animalis, L. fermentum, L. reuteri* (Axelsson, Ahrne *et al.* 1988; Fons, Hege *et al.* 1997; Gevers, Danielsen *et al.* 2003; Martel, Meulenaere *et al.* 2003). Moreover, the gene coding for the bifunctional aminoglycoside-modifying enzyme AAC(6')-APH(2") was detected in *L. salivarius* and *L. acidophilus* (Tenorio, Zarazaga *et al.* 2001) and chloramphenicol resistance gene *cat* was identified in *L. reuteri* (Lin, Fung *et al.* 1996). Obligate and facultative heterofermentative lactobacilli, and *L. salivarius,* are intrinsically resistant to vancomycin and other glycopeptide antibiotics.

Several genetic determinants for antibiotic resistance in *Lactobacillus* are harboured by extrachromosomal elements (Lin, Fung *et al.* 1996; Danielsen 2002; Gevers, Danielsen *et al.* 2003; Gfeller, Roth *et al.* 2003; Huys, D'Haene *et al.* 2006). However, transferable elements encoding resistances of clinical relevance, such as to the glycopetides have been excluded for some probiotic *L. reuteri* and *L. rhamnosus* strains (Klein, Hallmann *et al.* 2000).

Livestock. No report can be found on safety concerns related to lactobacilli in animals

4.4 Can the safety concerns be excluded?

There are apparently no specific safety concerns regarding a number of *Lactobacillus* species which have a long history of apparent safe use in the food chain. Susceptibility to antibiotics should be assessed as defined by the EFSA opinion for each strain (EFSA 2005).

4.5 Units proposed for QPS status

Due to the long history of safe use the following species are proposed for QPS status:

L. acidophilus, L. amylolyticus, L. amylovorus, L. alimentarius, L. aviaries, L. brevis, L. buchneri, L. casei, L. crispatus, L. curvatus, L. delbrueckii, L. farciminis, L. fermentum, L. gallinarum, L. gasseri, L. helveticus, L. hilgardii, L. johnsonii, L. kefiranofaciens, L. kefiri, L. mucosae, L. panis, L. paracasei, L. paraplantarum, L. pentosus, L. plantarum, L. pontis, L. reuteri, L. rhamnosus, L. sakei, L. salivarius, L. sanfranciscensis and *L. zeae.*

5 *Lactococcus*

The genus *Lactococcus* was previously known as group N-streptococci. The type species, *Lactococcus lactis*, (formerly *Streptococcus lactis*) is a well known dairy starter organism and members of this species are common component of cheese bacterial communities. The other recognised species do not have a history of intentional food use.

5.1 Taxonomic unit defined

As for other lactic acid bacteria, lactococci belong to the phylum *Firmicutes*. They are coccoid, non-motile organism metabolising hexoses homofermentatively and pentoses heterofermentatively. They are mesophilic with temperature optima generally at 30°C or less (for a review, see Axelsson 2004). The genus consists of five species: *Lactococcus lactis* (with subspecies *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* and *L. lactis* subsp. *hordniae*), *L. garvieae*, *L. plantarum, L. raffinolactis,* and *L. piscium* (Schleifer 1987; Williams, Fryer *et al.* 1990).

5.2 Is the body of knowledge sufficient?

The characteristics and habitat of the dairy species of *L. lactis* are well known, and they are extensively used as starters for the production of cheese and fermented milks. Complete genomic sequences of several industrial and laboratory strains are available (see Morelli 2004 for review). Outside the dairy environment natural habitats of lactococci include plant material (Kelly, Davey *et al.* 2000) and fish (Ringø 2004). *L. garviae* and *L. piscium* are well-known as fish pathogens (Williams, Fryer *et al.* 1990; Eyngor, Zlotkin *et al.* 2004).

5.3 Are there safety concerns?

Humans. *Lactococcus lactis* is consumed in large quantities in cheese and fermented milks by people of all ages, ethnic groups and variable health status with apparently no ill effects. Indeed, the relatively low growth temperature optima make even opportunistic infections unlikely. However, rare cases of endocarditis (Wood, Jacobs *et al.* 1955; Mannion and Rothburn 1990; Pellizzer, Benedetti *et al.* 1996; Halldorsdottir, Haraldsdottir *et al.* 2002) have been reported as well as septicemia (Durand, Rousseau *et al.* 1995) necrotising pneumonitis (Torre, Sampietro *et al.* 1990), septic arthritis (Campbell, Dealler et al. 1993), cerebral abscess (Akhaddar, El Mostarchid *et al.* 2002) and liver abscess (Nakarai, Morita *et al.* 2000). In the majority of these cases there have been predisposing factors, such as underlying disease, immunocompromised status or early age, although liver abscess caused by *L. lactis* in an immunocompetent adult has recently been reported (Antolin, Ciguenza *et al.* 2004). However, these infections represent extremely rare individual cases, and should not be regarded as an indication of human pathogenicity taking into account the extent of exposure to these microorganisms.

In general, dairy lactococci are sensitive to most clinical antibiotics to a degree that antibiotic residues in milk can cause starter failures. According to the results of (de Fabrizio 1994) strains of *L. lactis* were sensitive to ampicillin and other β-lactams (oxacillin, penicillin, pipericillin, and certain cephalosporins), chloramphenicol, erythromycin, amikacin, gentamicin, tetracycline, sulphonamide, trimethoprim/sulfamethoxazole and vancomycin. Somewhat lowered susceptibility towards carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin, and intrinsic resistance towards colistin, fosfomycin, pipedimic acid and rifamycin were observed.

Lactococci are well known to contain plasmids and to exchange genetic material by intra- and intergeneric conjugation (see Morelli 2004 for a review), and the potential for the spread of transferable antibiotic resistances thus exists. Indeed, pK214, a plasmid from a *L. lactis* strain originally isolated from a raw milk soft cheese, harbours resistance determinants for streptomycin (streptomycin adenylase), tetracycline (Tet S) and chloramphenicol (chloramphenicol acetylase) and an efflux protein conferring resistance to macrolides in *Escherichia coli*, but not in the strain itself (Perreten, Schwarz *et al.* 1997). Since all these resistance determinants showed homologies to genes resident in other species (*Streptococcus pyogenes*, *Staphylococcus aureus* and *Listeria monocytogenes*) pK24 illustrates the potential for genetic exchange even between taxonomically distant species.

Livestock. *Lactococcus garviae* and *L. piscium*, are well known fish pathogens (Williams, Fryer *et al.* 1990; Torranzo 2005), the former dominating in the warm water species and the latter at temperatures below 15°C. Moreover, *L. garviae* was originally isolated from the udder of a mastitic cow (Collins, Farrow *et al.* 1983). Thus these species have a distinct pathogenic potential in aquaculture, and maybe also in other livestock.

5.4 Can the safety concerns be excluded?

With the dairy lactococci the possibility of human or veterinary infections, at least in warm blooded animals, are extremely remote, and in practice this is not a concern. Thus with the dairy strains the main safety issue is the presence of acquired antibiotic resistances, which should be monitored.

With the other lactococcal species there is a lack of history of intentional food use, and the pathogenicity of certain species (*L. garviae*, *L. piscium*) in fish necessitates strain-specific safety assessment in eventual applications related to aquaculture.

5.5 Units proposed for QPS status

The dairy species of *L. lactis* (*L. lactis* subsp. *lactis*, its biovariant *diacetylactis*, and *L. lactis* subsp. *cremoris*) have a long history as dairy starters and an excellent safety record. The occasional and extremely rare infections, in which also these organisms have been associated, do

not warrant specific safety concerns. Thus these subspecies can be proposed for QPS status, provided that the presence of acquired antibiotic resistance determinants has been excluded (EFSA 2005).

6 *Leuconostoc*

The *Leuconostoc* genus contains obligate heterofermentrative lactic acid cocci. These bacteria have as their predominant natural habitat plants and fermented food of plant origin. Due to their positive contribution, members of this genus are frequently used as a starter cultures to promote food fermentation and malolactic fermentation in wine. Moreover, *Leuconostoc* species play a significant role in dairy fermentation, where they contribute to the aroma formation, and also in meat fermentation.

6.1 Taxonomic unit defined

Bacteria of the genus *Leuconostoc* belong to phylum *Firmicutes*. They are coccoid, non-motile organisms, showing obligate heterofermentative metabolism. They are mesophilic with temperature optima generally at 30°C or less. The genus consists currently of the following species: *L. carnosum, L. citreum, L. durionis, L. fallax, L. ficulneum, L. fructosum,, L. gelidum, L. inhae, L. kimchii, L. lactis, L. mesenteroides* subsp *cremoris, L. mesenteroides* subsp *dextranicums, L. mesenteroides* subsp *mesenteroides, Leuconostoc pseudoficulneum L. pseudomesenteroide.*

6.2 Is the body of knowledge sufficient?

The characteristics and habitat of the *Leuconostoc* are well known, and some species have a long history of apparent safe use as starter cultures for dairy, wine and vegetable fermentations. Moreover, they are among the dominant populations in microbial communities of several traditional fermented foods. Projects to determine the nucleotide sequence of the genome sequencing projects of *L. mesenteroides* are ongoing.

6.3 Are there safety concerns ?

Humans. *Leuconostoc* have generally been considered to be non-pathogenic bacteria. However, there have been reports that these organisms might occasionally be involved in human disease (Vagiakou-Voudris, Mylona-Petropoulou *et al.* 2002; Kumudhan and Mars 2004). Infections by *Leuconostoc* species are uncommon, and usually affect patients with an underlying disease, therefore *Leuconostoc* species described so far can rightly be considered to be non-pathogenic to humans.

Although there are few studies on the antibiotic resistance of *Leuconostoc*, strains from food isolates were also found carrying antibiotic resistance genes (Wang, Manuzon *et al.* 2006).

Livestock. No report can be found on safety concerns related to *Leuconostoc* species in animals.

6.4 Can the safety concerns be excluded?

There apparently are no specific safety concerns regarding *L. citreum, L. lactis*, and *L. mesenteroides,* which have a long history of apparent safe use in the food chain. Susceptibility to antibiotics should be assessed as defined by EFSA for each strain (EFSA 2005).

6.5 Units proposed for QPS status

Due to the long history of safe use, *L. citreum, L. lactis*, and *L. mesenteroides* species are proposed for QPS status.

7 *Pediococcus*

Pediococci are Gram-positive, coccus-shaped, lactic acid bacteria, showing the distinctive characteristic of formation of tetrads of cells, via cell division in two perpendicular directions in a single plane. Pediococci have been isolated from a variety of food fermentations, such as cheese, sausages and fermented plant products. Pediococci are also involved in spoilage of wine, beer and other alcoholic beverages. Due to their positive role in food fermentation, members of this genus are used as starter cultures for dairy, meat and vegetable fermentations.

7.1 Taxonomic unit defined

As for other lactic acid bacteria, pediococci belong to phylum *Firmicutes*. Phylogenetically all species of *Pediococcus* fall within the *Lactobacillus* cluster of lactic acid and related Gram-positive bacteria and forms a *Lactobacillus casei – Pediococcus* sub-cluster. The genus consists currently of the following nine species: *P. acidilactici*, *P. cellicola, P. claussenii, P. damnosus, P. dextrinicus, P. inopinatus, P. parvulus, P. pentosaceus* and *P. stilesii.*

7.2 Is the body of knowledge sufficient?

The characteristics and habitat of the species of the *Pediococcus* genus are well known, and some species have a long history of apparent safe use as starter cultures for dairy, sausage and vegetable fermentations. Moreover, they are among the dominant populations in microbial communities of several traditional fermented foods. Projects to determine the nucleotide sequence of *P. pentosaceus* are ongoing (http://genome.jgi-psf.org/draft_microbes/pedpe/pedpe.home.html).

7.3 Are there safety concerns?

Pediococci are consumed in large quantities in cheese and fermented sausages by people of all ages, ethnic groups and health status with apparently no ill-effects. Pediococci have generally been considered to be non-pathogenic. *Pediococcus* spp. are rarely isolated from clinical specimens, and there are few reports on their involvement in human disease (Heinz, von Wintzingerode *et al.* 2000), usually only affecting patients with an underlying disease. Therefore pediococci can be considered to be non-pathogenic to humans.

Acquired genes for antibiotic resistance have been detected in *Pediococcus* genus: *tet*(M) and *tet*(S) genetic determinants for tetracycline resistance have been found in *P. pentosaceus* (Gevers, Danielsen *et al.* 2003). Tankovic, Leclercq *et al.* (1993) reported the presence of erythromycin resistance determinants, homologous to *ermAM*, carried by a 46-kb plasmid, pVM20 in *P. acidilactici*. Moreover, the gene coding for the bifunctional aminoglycoside-modifying enzyme AAC(6')-APH(2") was detected in *Pedioccoccus acidilactici* (Tenorio, Zarazaga *et al.* 2001).

7.4 Can the safety concerns be excluded?

There are apparently no specific safety concerns regarding *P. acidilactici, P. dextrinicus* and *P. pentosaceus,* which have a long history of apparent safe use in the food chain. Susceptibility to antibiotics should be assessed as defined by EFSA for each strain (EFSA 2005).

7.5 Units proposed for QPS status

Due to the long history of safe use, the species *P. acidilactici, P. dextrinicus* and *P. pentosaceus* are recommended for QPS status.

8 Propionibacteria

Propionic acid bacteria have a long history of use as aroma producers in dairy products, and they have also been used as silage inoculants, due to the antifungal properties of propionic acid. The genus can be divided into dairy species and species associated with human or animal skin.

8.1 Taxonomic unit defined

Propionic acid bacteria (PAB) belong to the Actinomyctes-branch of *Firmicutes*.. They are mesophilic, non-sporing, non-motile irregular rods. The metabolism is anaerobic and the main fermentation endproducts are propionic acid, acetic acid and CO_2. PAB can be divided into dairy species (DPAB) and mucocutaneous PAB. The former include: *P. acidopropionici, P. australiense, P. cyclohexanicum, P. freudenreichii* subsp. *freudenreichii, P. freudenreichii*

subsp. *shermanii*, *P. jensenii, P. thoenii* and *P. microaerophilum.* The cutaneous species are *P. acnes, P. avidum, P. granulosum, P. lymohophilum* and *P. propionicum* (For a review, see Ouwehand 2004).

8.2 Is the body of knowledge sufficient?

DPAB have been traditionally associated with certain types of cheese (Swiss cheese or Emmenthal being the best known), although they have been occasionally isolated from rumen and as contaminants from spoiled foodstuffs. The mucocutaneous PAB belong to the normal microbiota of human skin and/or mucous membranes, and can occasionally be isolated from the faeces.

The traditional use of DPAB is as cheese starters. Their main function is to produce propionic acid, which is an important aroma component and to form the characteristic "eyes" in cheese through development of CO_2. Propionic acid has also antimicrobial properties, particularly against fungi. In addition PAB are known to produce a variety of bacteriocins (Ouwehand 2004), such as acnecin, jenseniins P and G, propionicins PLG-1, T1 SM1 and SM2, and protease-activated antimicrobial peptide (PAMP). The producing species are mainly cutaneous strains, although strains of *P. freudenreichii* subsp. *shermanii* are also known to produce acnecin. The antimicrobial spectrum of these bacteriocins cover other PAB, lactic acid bacteria, other Gram-positive and Gram-negative bacteria as well as yeasts and filamentous fungi in some cases.

Because of their antimicrobial action DPAB are also used as components of protective cultures that are used to prevent microbial spoilage of foodstuffs (Ayres 1992; Mäyrä-Mäkinen 1995). The use of PAB as silage starters is also mainly based on their antifungal action.

8.3 Are there safety concerns?

Humans: Although mucocutaneous PAB are normal commensals on human skin or mucous membranes, they, especially *P. acnes* and *P. propionicum,* have been also associated with the pathogenesis of acne (Bojar and Holland 2004) and various other infections (Al-Mazrou 2005; Lutz, Berthelot *et al.* 2005). *P. propionicum,* possibly better known among infectious disease under its previous designations "*Actinomyces propionicus*"or "*Arachnia propionica*", is one of the less frequent, but nonetheless typical causes of human actinomycosis, a disease which has been known for decades for its notorious diagnostic and therapeutic problems. Furthermore, *P. propionicum* is the most characteristic and frequent causative agent of human lachrymal canaliculitis with and without conjunctivitis (Brazier and Hall 1993).

The complete genomic sequence of the organism *P. acnes* is available, and might eventually provide the determinants of pathogenicity allowing these species to be differentiated from DPAB (Bruggemann 2005).

Due to their use as cheese starters, relatively large amounts of DPAB are consumed by humans in Europe without any observable ill effects. Toxicological studies on *P. freudenreichii* subsp. *shermanii* have been reported. Intraperitoneal doses of $10^9 - 10^{10}$ cfu did not cause observable ill effects in mice, guinea pigs or rabbits, nor did it have any cytotoxic effect on tissue cultures (Sidorchuk and Bondarenko 1984).

The information on antibiotic resistance of Propionibacteria is limited to mucocutanoeous species, in particular to *P. acnes, P. granulosum,* and *P. avidum* and *P. propionicum*, and to antibiotics used either topically or systematically to combat infections. Tetracycline and erythromycin resistances have been mainly associated with base change mutations in 16S rDNA. A transposon-associated erythromycin resistance determinant conferring resistance to MLS antibiotics has also been characterised among the clinical isolates (Ross, Snelling *et al.* 2001; Ross, Snelling *et al.* 2003). The presence of acquired determinants for antimicrobial resistance has not been reported in *P. freudenreichii.*

Livestock: No reports of veterinary problems associated with the use of DPAB in silage could be found. However, there is an Australian report of four cases of abscess-like gross lesions in carcases of cattle, where the associated microorganism has been characterised as closely related to *P. cyclohexanicum/P.freudenreichii* cluster (Forbes-Faulkner, Pitt *et al.* 2000).

8.4 Can the safety concerns be excluded?

There is a long history of apparent safe use of DPAB, particularly *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* in dairy foods and to certain extent also in silage. Although a closer inspection might reveal occasional cases of opportunistic infections associated with microorganisms closely related to these bacteria, these do not warrant specific safety evaluation of taxonomically well characterised *P. freudenreichii* and its subspecies or closely related dairy species.

Since the data from mucocutaneous strains show that acquired antibiotic resistance can be associated with Propionibacteria, susceptibility to antibiotics should be assessed as defined by EFSA for each strain (EFSA 2005).

8.5 Units proposed for QPS status

There is a long history of safe use of DPAB, particularly *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* in dairy foods and to certain extent also in silage. Occasional cases of opportunistic infections associated with microorganisms closely related to these bacteria may be found but these do not warrant specific safety evaluation of taxonomically well characterised *P. freudenreichii* and its subspecies. Consequently, *P. freudenreichii* is proposed for QPS status, provided that the absence of acquired antibiotic resistances is demonstrated as indicated in the EFSA opinion (EFSA 2005).

9 *Streptococcus thermophilus*

S. thermophilus is a relevant dairy starter microorganism, used for the manufacture of fermented milks and cheese. This species belongs to the genus *Streptococcus*, a taxonomical unit which includes pathogenic and oral streptococci. Since *S. thermophilus* is an exception in this genus, for its relevant role as industrial and food organism, only this species has been considered for the QPS assessment.

9.1 Taxonomic unit defined

The taxonomy of *S. thermophilus* has been quite controversial, it has been considered a subspecies of *S. salivarius* and more recently this species has been revived on the basis of molecular taxonomy (Schleifer 1991).

9.2 Is the body of knowledge sufficient?

S. thermophilus is widely used as starter cultures for cheese and fermented milk. The biology of this microorganism is well known and three different genomes sequences are available (Hols, Hancy *et al.* 2005).

9.3 Are there safety concerns?

S. thermophilus is consumed in large quantities in cheese and fermented milks by people of all ages, ethnic groups and variable health status with apparently no ill effects. This species has generally been considered to be non-pathogenic and no safety concerns are envisaged.

Although there are few reports on the antibiotic resistance/susceptibility in *S. thermophilus*, acquired resistance genes has been detected in this species. Thus, genetic determinants for tetracycline *tet*(S) and erythromycin *erm*(B) resistance were detected in dairy strains of *S. thermophilus* (Wang, Manuzon *et al.* 2006).

9.4 Can the safety concerns be excluded/Qualifications?

Since there are not safety concerns the only qualification required is the assessment of susceptibility to antibiotics for each single strain, as defined by EFSA.

9.5 Units proposed for QPS status

There are no safety concerns for *S. thermophilus,* therefore QPS status is proposed for this species, provided that the lack of acquired antibiotic resistance is demonstrated (EFSA 2005).

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APPENDIX B. Scientific report on the assessment of Bacillus species

For decades, strains belonging to several species of *Bacillus* have been deliberately introduced into the food chain either as plant protection products or as an animal feed supplement. The knowledge gained from this use suggests that, for some species at least, their safety could be assured by the "Qualified Presumption of Safety" (QPS) approach, according to EFSA (2005).

1 – Identity of QPS unit.

Since the first edition of the Bergey's Manual of Systematic Bacteriology, the structure and content of the genus *Bacillus* have been substantially modified (Claus 1986; Ash, Priest et al. 1993; Priest 1993). In particular, several former *Bacillus* species have been excluded from the genus and reallocated to new genera.

Most strains of Gram-positive, spore forming bacteria that have been or are used as animal feed supplements or plant protection products belong to species included in the new restricted definition of the genus *Bacillus* (Reva 2004; Hong, Duc le et al. 2005). The list of species included in this genus can be found in the second edition of the Bergey's Manual of Systematic Bacteriology, Volume 3, or in http://www.bacterio.cict.fr/.

The present document addresses species of the *B. subtilis* group that are, or were previously, classified as *B. subtilis* (*B. amyloliquefaciens, B. atrophaeus, B. mojavensis, B. subtilis* and *B. vallismortis*), and selected species within the *B. cereus* group (*B. cereus, B. mycoïdes, B. pseudomycoides, B. thuringiensis* and *B. weihenstephanensis*), since an extensive body of knowledge is available for these two groups either as the former species, or as the more recent, restricted species. Other species that that have been notified to EFSA, and for which a sufficient body of knowledge exists, are also considered (*B. clausii, B. coagulans B. fusiformis, B. lentus B. licheniformis, B. megaterium, B. pumilus* and *Geobacillus stearothermophillus).*

The species listed above can be reliably identified using a 16S rRNA gene sequence. For species of the *B. subtilis* group the sequence of the gyraseA gene discriminates strains of the constituent species (Chun and Bae 2000). The species within the *B. cereus* group are difficult to distinguish reliably. However, *B. cereus* (including *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis*) do not synthesise a parasporal crystal protein, and can thereby be distinguished from the crystalliferous *B. thuringinesis*.

2 - Is the body of knowledge sufficient?

Several strains belonging to the species *B. clausii, B. coagulans, B. licheniformis, B. megaterium, B. pumilus, B. subtilis and B. cereus* (excluding *B. anthracis*) have been used as probiotics, animal feed supplements or in aquaculture (SCAN 2000; Hong, Duc le et al. 2005). Furthermore, several *Bacillus* species are involved in the preparation of traditional fermented dishes in Africa and Asia (Sarkar, Hasenack et al. 2002). Strains of *B. thuringiensis*, a species which belongs to the *B. cereus* group and is characterised by the production of insect toxic proteins which form a parasporal crystal in the bacterial cell, has a long history of use as plant protection products. Strains of *B. cereus* (Halverson and Handelsman 1991) and of *B.subtilis* (Krebs 1998; Kahn 2002; Cavaglieri, Orlando et al. 2005) have been used for treatment of seeds and roots to protect or promote the growth of plants. Several *Bacillus* species, as well as *Geobacillus stearothermophilus*, which does not grow below 45°C, are major sources of commercial enzymes (SCAN 2000). No foodborne cases or food safety problems have been linked to these usages of *Bacillus* strains so far, although it can be noted that *B. thurigiensis* strains, identical to those present in commercial plant protection products, have been isolated from faecal samples of greenhouse workers after exposure to such products (Jensen, Larsen et al. 2002).

Annotated genome data are currently available for several strains within the species *B. clausii, B. cereus, B. licheniformis, B. subtilis, B. thuringiensis*, and *Geobacillus kaustophilus* thereby contributing significantly to the body of knowledge and decreasing the probability that unforeseen hazards could be associated with these bacilli (http://www.cbs.dtu.dk/services/GenomeAtlas/).

3 - Are there safety concerns?

3.1 Human safety concerns

3.1.1 Anthrax

The highly toxic bacterium *Bacillus anthracis*, the cause of anthrax in humans and animals, is a member of the *Bacillus cereus* group, and other members of the group have been seen to carry genes encoding anthrax-like toxins (Hoffmaster, Ravel et al. 2004).

3.1.2 Food poisoning

Several of the *Bacillus* species used as animal feed supplements, probiotics, plant protection products or seed coating agents are also known as agents of food poisoning (Kramer 1989; Granum 2000; EFSA 2005).

B. cereus is a frequent agent of foodborne diseases. It is the cause of two distinct kinds of poisoning, (i) an emetic intoxication caused by the ingestion of the toxin cereulide, which is preformed in the food (Granum 2001), and (ii) a diarrhoeal infection caused by the production of either of the enterotoxins hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) or

cytotoxin K (CytK) in the small intestine. Also *B. thuringiensis* has been implicated in cases of food poisoning (Jackson, Goodbrand et al. 1995) and most strains of *B. thuringiensis* produce the diarrhoeal *B. cereus* enterotoxins (Gaviria Rivera, Granum et al. 2000; Hansen and Hendriksen 2001). The diagnostic methods that are currently used to identify the cause of food poisoning often do not distinguish between *B. cereus* and *B. thuringiensis*. Therefore, some of the clinical cases attributed to *B. cereus* may actually have been caused by strains of *B. thuringiensis*.

Strains of *B. fusiformis, B. licheniformis, B. mojavensis, B. pumilus,* and *B. subtilis* are known as rare causes of foodborne poisoning (Kramer 1989). A few strains among these species produce cytotoxins (Salkinoja-Salonen, Vuorio et al. 1999; From, Pukall et al. 2005). Strains of other species have been reported to produce *B. cereus*–like toxins, although the identification of the organisms in these studies is uncertain (*B. amyloliquefaciens, B. circulans*, *B. lentimorbis, B. lentus,* and *B. megaterium*).

3.1.3 Other clinical implications

B. cereus have been responsible for severe, although rare clinical infections in humans (Teyssou 1998): endophtalmitis (Callegan, Engelbert et al. 2002; Callegan, Cochran et al. 2006), necrotizing infections (Darbar, Harris et al. 2005), endocarditis (Cone, Dreisbach et al. 2005), bacteremia (Hernaiz, Picardo et al. 2003), osteomyelitis (Popykin 2002), septicemia (Matsumoto, Suenaga et al. 2000), pneumonia (Miller, Hair et al. 1997; Gray, George et al. 1999) and liver abscess (Latsios, Petrogiannopoulos et al. 2003).

3.1.4 Antibiotic resistance

Resistance to macrolides has been observed in several cases in species belonging to the genus *Bacillus*. The most commonly found mechanism is encoded by the *erm*(D) gene (Gryczan, Israeli-Reches et al. 1984; Hue and Bechhofer 1992; Kim, Choi et al. 1993), but the genetic location and potential transferability of this gene has not been determined. However, resistance genes present on extra-chromosomal elements of bacilli include plasmid-encoded *erm*(C), which has been identified in *B. subtilis* (Monod, Denoya et al. 1986), plasmid encoded *tet*(L), which has been found in *B. stearothermophilus* and *B. subtilis* (Hoshino, Ikeda et al. 1985; Sakaguchi, Amano et al. 1988), as well as mobilisable plasmid-encoded tetracycline resistance of the *B. cereus* group organisms (Battisti, Green et al. 1985). Considering that many examples of highly potent mechanisms of conjugative plasmid transfer have been described for the genus *Bacillus* (Andrup, Smidt et al. 1998; Thomas, Morgan et al. 2001; Poluektova, Fedorina et al. 2004), there is a high probability that such plasmids mobilise other plasmids that encode resistance. Additionally, in *B. cereus* the *tet*(M) tetracycline resistance determinant has been found on the conjugative transposon Tn*916*, which is known to have a very broad host-range (Agerso, Jensen et al. 2002), and also *B. subtilis* is known to harbour conjugative transposons that encode tetracycline resistance (Roberts, Pratten et al. 1999).

3.1.5 Livestock safety concerns

B. cereus has been implicated in rare cases of bovine mastitis, bovine and ovine abortion (Parkinson, Merrall et al. 1999; Rowan, Caldow et al. 2003). *B. licheniformis* has been responsible for rare cases of bovine and ovine abortions (Agerholm, Willadsen et al. 1997; Rowan, Caldow et al. 2003).

4 – Can the safety concern be excluded?

4.1 Human safety concerns

4.1.1 Anthrax

B. anthracis, which is obviously not suited for QPS status, cannot be distinguished easily from the other members of the *B. cereus* group by 16S rRNA sequencing (Sacchi, Whitney et al. 2002).

However, absence of genes encoding anthrax-like toxins in species belonging to the *B. cereus* group can be verified by PCR (Cheun, Makino et al. 2003; Ryu, Lee et al. 2003). Additionally, absence of expression of the toxins can be verified by IP injection in mice.

4.1.2 Food poisoning

Within the genus *Bacillus*, food poisoning is clearly linked to the production of emetic toxins or to the production of the diarrhoeal enterototoxins Nhe, Hbl and CytK (Granum 2001; EFSA 2005).

Emetic toxins (cyclic peptides) are heat stable, resists pasteurisation and most industrial food thermal sterilisation processes, and are produced at temperature between 12 and 32°C (From, Pukall et al. 2005). They are formed by bacilli present in food products prior to ingestion.

Production of the emetic toxin cereulide has generally been believed to be restricted to a group of very closely related strains within the *B. cereus* group (Ehling-Schulz, Svensson et al. 2005), but was recently found in also in *B. weihenstephanensis* (Thorsen, Hansen et al. 2006). The cereulide biosynthetic gene cluster is located on a large plasmid, which may be subject to lateral transfer (Hoton, Andrup et al. 2005; Ehling-Schulz, Fricker et al. 2006). However, *Bacillus* strains encoding the cereulide biosynthetic genes can be identified by PCR (Ehling-Schulz, Fricker et al. 2004). Additionally, production of cereulide can be rapidly detected in cultures grown on agar media by its ability to inhibit sperm mobility (Andersson, Mikkola et al. 1998; Haggblom, Apetroaie et al. 2002; From, Pukall et al. 2005).

Emetic intoxication caused by *Bacillus* species other than *B. cereus* is very uncommon and the body of knowledge on toxigenic strains is not as large as for *B. cereus*. However, strains producing toxins with emetic activity can be identified by the same assays as used for *B. cereus* (From, Pukall et al. 2005).

Diarrhoeal enterotoxins are produced in the mammalian gut after ingestion of strains of certain *Bacillus* species. Formation of enterotoxins is largely restricted to the *B. cereus* group, but has occasionally been reported in other members of the genus *Bacillus*.

Strains of *B. cereus* involved in diarrhoeal cases tend to produce high level of enterotoxins (Guinebretiere, Broussolle et al. 2002). The cytotoxic activity on epithelial cells of culture supernatants is therefore a good indication of their ability to cause foodborne poisoning (SCAN 2000). Strains that have the potential to cause diarrhoea can thus be identified by the toxicity of culture supernatants on Vero cells or other epithelial cell cultures (Sandvig and Olsnes 1982; SCAN 2000). The strains produce the *B. cereus*-like enterotoxins at 37° C, with the exception of *B. weihenstephanensis*, which produces the toxins only at <30° C.

Even though the production of enterotoxins can thus be addressed and eventually excluded, the fact that strains within the *B. cereus* group are well known agents of food poisoning makes QPS inapplicable for this group of strains. Current knowledge shows that the vast majority of *B. cereus* strains are toxin producers, and thus cannot meet the qualifications required for all strains within the *Bacillus* genus.

4.1.3 Other clinical implications

When identified, the causes of the non-gastrointestinal *B. cereus* infections in human, described in section 3.1.3, were mostly wounds, trauma, intravenous drug usage (Callegan, Engelbert et al. 2002; Popykin 2002; Darbar, Harris et al. 2005). Some infections were nosocomial (Gray, George et al. 1999; Matsumoto, Suenaga et al. 2000) and some occurred in immuno-compromised patients (Motoi, Ishida et al. 1997; Cone, Dreisbach et al. 2005). Even though relation with foods or the food chain seems very unlikely, it is concluded that the potential risks associated with the use of *B. cereus* are too many for this strain to be included on the QPS list.

The virulence factors involved in infection by *Bacillus* spp. outside of the gastrointestinal tract are not as well known as for food poisoning. *Bacillus* spp. other than *B. cereus*, in particular some strains of *B. licheniformis, B. pumilus,* and *B. subtilis* produce very active cyclic biosurfactants with large potential medicinal and industrial applications (Mulligan 2005; Rodrigues, Banat et al. 2006). These cyclic biosurfactants are also haemolytic (Dufour, Deleu et al. 2005) and could be cytotoxic. The role of cytotoxins and of cytotoxic biosurfactant molecules in infections occurring outside the gastrointestinal tract caused by *Bacillus* spp. other than *Bacillus cereus* should be investigated further. This might provide a mean to discriminate strains with infectious potential.

4.1.4 Antibiotic resistance

Safety concerns associated with acquired resistance genes can be excluded as described in the opinion of the Scientific Panel on Additives and Products or substances used in Animal Feed (http://www.efsa.europa.eu/en/science/feedap/feedap_opinions/993.html)

4.2 Livestock safety concerns

The origin of mastitis and abortion caused by *B. cereus* has not been clearly established but does not seem to be linked to feed. In the case of abortion caused by *B. licheniformis* the origin of the infection is not identified. In one case, it was reported that placenta and the digestive tract content contained large number of *B. licheniformis* (Parvanta 2000). The body of knowledge available does not indicate that presence of these species in feed was a cause of infections outside of the gastrointestinal tract in animals.

(Rowan, Caldow et al. 2003) observed that all strains of *Bacillus* spp. implicated in mastitis and abortion in animals were cytotoxic on the Hep-2 epithelial cell line. As also mentioned in the context of human clinical concerns, the role of cytotoxins and of cytotoxic biosurfactant molecules in non gastro-intestinal livestock infections caused by *Bacillus* spp. other than *Bacillus cereus* should be investigated further.

4.3 Exceptions related to end-use

Safety concerns may be excluded when the use of the strain does not imply its entry into the food chain. This is the case for strains used as seed coating agents where the probability that the bacterial cells are transferred to the edible part of the crop is very low. *Bacillus* spp, including toxigenic strains, are naturally abundant in the soil and restricted use of well-described *Bacillus* strains producing either enterotoxins or cyclic peptides as seed coating agents only should not represent a threat to the environment.

It is likely that strains used as seed coating agents act in part by the production of compounds inhibiting plant pathogens, and such compounds may also have a toxic activity on epithelial cells or on sperm cells. Therefore, in the case of seed coating agents, excluding strains toxic to epithelial cells or to sperm cells is not necessary to ensure food safety and might be contradictory with the purpose of using such strains.

5 - Species proposed for QPS

In conclusion, it is proposed to include a number of *Bacillus* species notified to EFSA (*B. clausii, B. coagulans B. fusiformis, B. lentus B. licheniformis, B. megaterium, B. pumilus* and *Geobacillus stearothermophillus)*, and species previously or presently classified as *B. subtilis* (*B. amyloliquefaciens, B. atrophaeus, B. mojavensis, B. subtilis* and *B. vallismortis*) on the list of QPS granted units due to the substantial body of knowledge available about these bacteria. Since all bacteria within the listed species potentially possess toxigenic traits, absence of toxigenic activity needs to be verified for qualification.

Bacillus spp. belonging to the *Bacillus cereus sensu lato* group (*B. cereus sensu stricto, B. mycoïdes, B. pseudomycoides, B. thuringiensis* and *B. weihenstephanensis)* are not proposed for QPS, since it is known that the vast majority of strains within of this group are toxin producers and thus not meet the required qualifications.

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APPENDIX C: Scientific report on the assessment of Yeasts

Today, the impact of yeasts on food and beverage production extends beyond the original and popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae* (Querol, Belloch *et al.* 2003; Fleet 2006). Yeasts contribute to the fermentation of a broad range of other commodities, where various species may work in concert with bacteria and/or filamentous fungi. Many valuable food ingredients and processing aids are now derived by exploiting yeast properties, such as anti-fungal activity, enabling the yeasts to be used as novel agents in the biocontrol of food spoilage. The probiotic activity of some yeasts is another novel property that is of increasing interest especially in relation to animal feed. Finally, there are environmental aspects to be considered when yeasts are used as biocontrol agents.

Although yeasts are part of the microbiota of many foods and beverages they are rarely (if ever) associated with outbreaks or cases of food-borne illness.

1. Identity of the QPS unit

In addition to *S. cerevisiae*, *S. bayanus* and *S. pastorianus*, it is now well established that various species of *Candida*, *Hanseniaspora, Issatchenkia, Kluyveromyces, Metschnikowia, Pichia* and *Schizosaccharomyces* can make a positive contribution in the manufacture of fermented foods, dairy products, meats, cereals, coffee and sauces. The most frequently encountered and important species in dairy products are *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *S. cerevisiae*, *Galactomyces geotrichum*, *Candida celanoides* and various *Pichia* species. In the case of the fermentation of meat sausages and maturation of hams various species of *Debaryomyces*, *Yarrowia lipolytica* and various *Candida* species are involved. *S. cerevisiae*, *S. exiguous*, *C. humicola*, *C. milleri*, *C. kruseii, C. orientalis*, *Torulaspora delbrueckii* and various *Pichia* species are used in the fermentation of cereal products. The growth and activities of a diversity of *Hanseniaspora*, *Candida*, *Pichia*, *Issatchenkia*, *Kluyveromyces* and *Saccharomyces* species have been reported in the fermentation of coffee beans and cocoa beans. *Zygosaccharomyces rouxii*, *C. versatilis* and *C. etchellsii* are important osmotolerant species that play a key role in soy sauce fermentation (Boekhout 2003; Romano 2006). Moreover, baker's and brewer's yeasts (*S. cerevisiae*) have been available for many years as dietary supplements because of their high contents of B-group vitamins, proteins, peptides, amino acids and trace minerals.

In summary, some species of the genera *Candida*, *Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Saccharomyces, Schizosaccharomyces, and Xanthophyllomyces* can be included as the most relevant and commonly encountered yeasts

1.1. Taxonomy.

The identification, naming and placing of yeasts in their proper evolutionary framework are of importance to many areas of science including the food industry. At present, approximately 750 yeast species are recognised but only a few are frequently isolated. Several definitions have been used to describe the yeast domain. Thus, yeasts may be defined as being ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, with or without pseudohyphae and hyphae, and forming sexual states that are not enclosed in fruiting bodies (Boekhout 2003). In the last version of the "The yeasts; a taxonomic study" (Kurtzman 1999) there is an extensive taxonomic revision.

1.2. Methods for yeast identification.

Yeasts are commonly identified either phenotypically or, more recently, from diagnostic sequences. Methods based on phenotype, include fermentation reactions on a selected set of sugars and growth responses on various carbon and nitrogen sources or on other diagnostic compounds (Krejer-van Rij 1984; Barnett 2000). However, these characteristics can vary according to growth conditions and sometimes the species are defined by a unique physiological characteristic that is controlled by a single gene. By contrast, techniques using molecular biology are seen as an alternative to traditional methods since they analyse the genome independently of the physiological state of the cell (for a review see Boekhout 2003; Fernández-Espinar 2006). The nucleotide sequences of the domains D1 and D2 located at the 5' end of gene 26S (Kurtzman and Robnett 1998) and PCR amplification of ribosomal DNA regions and restriction of the gene 5.8S rRNA gene and the adjacent intergenic regions ITS1 and ITS2 are the molecular methods commonly used for the identification of yeasts (Fernández-Espinar 2006). These techniques are more reproducible and faster that the conventional methods based on physiological and morphological characteristics.

2. Is the body of knowledge sufficient?

Indigenous, also referred to as traditional, fermented foods are those popular products that since early history have formed an integral part of the diet and that can be prepared in the home or by cottage industries using relatively simple techniques and equipment. Some of these products have undergone industrial development and are now manufactured on a large scale. Yeasts occur in a wide range of fermented foods, made from ingredients of plant or animal origin. When yeasts are abundant they have a significant impact on food quality parameters such as taste, texture, odour and nutritional value. Although several products are obtained by natural fermentation, the use of traditional starter cultures is widespread.

The principal yeasts pathogenic for humans are *Candida albicans* and *Cryptococcus neoformans* which cause a range of mucocutaneous, cutaneous, respiratory, central nervous, systemic and organ infections (Hazen 2003; Richardson 2003). Usually, healthy, immunocompetent individuals are not at

risk of such infections. Generally, individuals with weakened health and immune function are at greatest risk, and include cancer and AIDS patients, hospitalised patients and patients who are administered immunosuppressive drugs, broad-spectrum bacterial antibiotics and radio- and chemotherapies. The increased frequency of such individuals in the community, has led to an increase in the reporting of yeast infections due to an increasing number of yeast species other than *C. albicans* and *Cryp. Neoformans* (Hazen 1995; Georgiev 2003; Hobson 2003; Richardson 2003). These include species that are frequently found in food such as *Candida krusei/orientalis*, *P. anomala*, *Kluy. Marxianus*, *S. cerevisiae* and various *Rhodotorula* species (Richardson 2003; Enache-Angoulvant and Hennequin 2005; Fleet 2006).

2.1. *Candida*

The genus *Candida* is the largest in number of species of the yeast genera, and is present in almost every environment. Yeasts of this genus are abundantly distributed in nature on land and sea, associated with animals or plants and inanimate objects.

2.1.1. Taxonomic unit defined

The genus *Candida* comprises 163 species; including anamorphic ascomycetous yeasts that reproduce by multilateral budding and which are not assigned to morphological unique genera. As a result this is a highly heterogeneous genus comprising species whose perfect states are still unknown. This genus is distributed across the ascomycetous yeast domain, overlapping with other genera according to phylogenetic analysis using ribosomal genes (Kurtzman and Robnett 1998).

This genus regroups a large number of species but the list can be refined to around 60 species that are present in food, the majority of them as spoilage organisms. A smaller number of species are used for food processing, as biocontrol agents (*e.g. C. glabrata* is used to control filamentous fungi in plants), or that are likely to be used by the industry and may cause opportunistic infections in humans. No literature pertaining to the use of *Candida* for animal feed can be found.

The best targets for the identification of species of this genus are the sequences D1/ D2 (26S) or PCR and restriction of 5.8S-ITS

The genome sequences are available for:

- *C. albicans* (http://genolist.pasteur.fr/CandidaDB)
- *C. glabrata* (http://cbi.labri.fr/Genolevures/elt/CAGL)
- *C. guilliermondii* (http://www.broad.mit.edu/)
- *C. dubliniensis* (http://www.genedb.org/genedb/cdubliniensis/)
- *C. lusitaniae* (http://www.broad.mit.edu/)
- *C. parapsilosis* (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/).
- *C. tropicalis* (http://www.broad.mit.edu/)

2.1.2. Is the body of knowledge sufficient?

Species of this genus can be found in food-processing environments and have been recovered as contaminants in a large number of foods, such as fruits, fruit juices, soft drinks, alcoholic beverages, products with high sugar content, vegetables and grains, salted and acid preserved foods, dairy products, meat and meat-derived products. The list of species that are commonly used in the food industry include: *C. zelanoydes,* which contributes to the flavour and texture during the maturation of cheese and in the production of fermented milks (kefir and koumiss), *C. milleri* for flavour and rheology in sourdough breads, *C. tropicalis*, *C. parapsilopsis* and *C. pelliculosa,* which occur in the wet fermentation of coffee, *C. etchellsii* and *C. versatilis,* which contribute to the flavour of soy sauce, *C. rugosa,* which is involved in cocoa fermentations, *C. utilis* (=*P. jadinii*) and *C. maltosa*, which are used for biomass production from carbohydrate and hydrocarbon substrates respectively, *C. oleophila* and *C. sake*, which are commercialised for use as fungal biocontrol agents.

2.1.3. Are there safety concerns?

Humans. The principal human pathogenic yeasts are species of *Candida*, such as *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parasilopsis*, *C. tropicalis*, *C. viswanathii* (Richardson 2003). The principal and most common pathogen in the genus is *C. albicans*; other *Candida* species are however actually considered as emerging pathogens such as *C. glabrata*, which is being reported by some European medical centres as the main cause of candidaemia, *C. parapsilosis,* which is frequently isolated from skin lesions, and *C. tropicalis,* which is the second most frequent yeast, after *C. albicans*, causing deep-seated mycosis disease. Recently, new emerging pathogenic yeasts, such as *C. dubliniensis,* have been described, associated with hyperalimentation, broad-spectrum antibiotics, and immunosuppressive or antineoplastic therapies. Increasingly, systemic infections caused by *C. guilliermondii* are being reported. In most cases, invasive *Candida* infection is thought to be endogenous in origin, but transmission of organisms from person to person can also occur.

Livestock. No information could be found regarding the use of *Candida* as animal feeds or probiotics.

2.1.4. Can the safety concerns be excluded?

The two main species of concern are *C. albicans* and *C. tropicalis*. *Candida* is involved in many cases of septicaemia (*e.g.* after surgery).

2.1.5. List of units proposed for QPS status

Although a number of *Candida* species associated with food appear not to cause infection, *viz. C. etchellsii*, *C. maltosa*, *C. milleri*, *C. oleophila*, *C. pelliculosa, C. rugosa*, *C. sake*, *C. utilis* (=*P.*

jadinii), *C. versatilis* and *C. zelanoydes,* the fact that the principal human pathogenic yeasts are species of *Candida*, and that more and more *Candida* species are today considered as emergent pathogens, makes the genus *Candida* unsuitable for QPS status.

2.2. *Debaryomyces*

The genus *Debaryomyces* comprises 15 species. Many representatives can be isolated from natural habitats such as air, soil, pollen, tree exudates, plants, fruits, insects, and faeces and gut of vertebrates.

2.2.1. Taxonomic unit defined

Nine of these *Debaryomyces* species: *D. carsonii*, *D. etchellsii*, *D. hansenii*, *D. maramus*, *D. melissophilus*, *D. polymorphus*, *D. pseudopolymorphus*, *D. robertsiae* and *D. vanrijiae*, have been found in a variety of processed foods; such as fruit juices and soft drinks, wine, beer, sugary products, bakery products, dairy products and meat or processed meats. The presence of *Debaryomyces* species in foods usually has no detrimental effects and in some cases is beneficial to the food. The taxonomy is well defined with precise description available. The best method for species identification is PCR-RFLP of the IGS region of rDNA (Quiros, Martorell *et al.* 2006).

The partial genome sequence of *D. hansenii* is available at (http://cbi.labri.fr/Genolevures/about/GL1_genome.php)

2.2.2. Is the body of knowledge sufficient?

Humans. Some *Debaryomyces* species are important in the ripening of fermented food products such as cheese and meat products. Where *D. hansenii* is used in the ripening of cheeses they metabolise lactic acid, raising the pH to allow the growth of proteolytic bacteria, and the yeast exhibits lipolytic activity that contributes to the development of cheese aromas. Proteolytic and lipolytic activities of *D. hansenii* have been described in the curing of ham and ripening of sausages and their presence in salami influences the red coloration and improves the quality of the product. Nevertheless, excessive growth of *Debaryomyces* species may cause undesirable sensory changes due to the formation of off-odours and off-flavours. These species have also been found as frequent contaminants of spoiled yoghurts, ice creams, fish, shellfish, *etc.*

Livestock. No information could be found regarding the use of *Debaryomyces* as animal feeds or probiotics.

2.2.3. Are there safety concerns?

The main species of *Debaryomyces* used in food processing is *D. hansenii*, the anamorph form of which is *Candida famata*. *C. famata* has been repeatedly associated with catheter-related bloodstream infections, and occasionally with infections of the central nervous system. The reservoir of *C. famata* is not known but there is a possibility that nosocomial infections can occur via air contamination (Wagner, Sander *et al.* 2005).

No studies on antifungal susceptibility of *Debaryomyces* are available.

2.2.4. List of units proposed for QPS status

It is proposed to grant *D. hansenii* QPS status.

2.3. *Hanseniaspora*

Hanseniaspora species are mainly found in the soil, on fruits and trees and in spoiled foods and beverages. Members of this genus are characterised by apiculate cells with vegetative reproduction by bipolar budding in basipetal succession. The six species in the genus that have valid descriptions are physiologically very similar; they ferment glucose, assimilate a few carbon compounds (arbutin, cellobiose, glucose, glucono-δ-lactone and salicin), and require inositol for growth. However, they show marked differences in the shape and number of ascospores, a criterion used for species identification.

2.3.1. Taxonomic unit defined

According to ribosomal sequences (26 S and 5.8S genes) the six species included in this genus are monophyletic and can be divided into two subgroups. This subdivision was supported by electrophoretic chromosome patterns. *Hanseniaspora guilliermondii*, *H. uvarum* and *H. valbyensis* have 8 to 9 chromosomes, while the second group comprises the species *H. occidentalis*, *H. osmophila* and *H. vineae* that have only 5 chromosomes. The anamorphic form of this genus is the well known *Kloeckera*.

The best targets for identifying species of this genus are the sequences D1/ D2 (26S) or PCR and restriction of 5.8S-ITS

2.3.2. Is the body of knowledge sufficient?

Humans. The species are most frequently isolated from soil, fruits and plant exudates. The species occur on grapes and processed fruit. *H. uvarum*, the most relevant species of this genus, is important

in the first phase of grape fermentation and is supposed to play a role in the production of certain flavours beneficial for the quality of wine and cider. Little is known regarding the other species.

Livestock. No information could be found regarding the use of *Hanseniaspora* as animal feeds or probiotics.

2.3.3. Are there safety concerns?

No data concerning this yeast causing opportunistic infections have been found.

2.3.4. List of units proposed for QPS status

H. uvarum is proposed for QPS status

2.4. *Kluyveromyces*

The genus *Kluyveromyces* has a very wide distribution; its representatives have been recovered from substrates as diverse as seawater, soil, insects, plant material, fresh fruit, jams and other fruit preserves, dairy and bakery products, and have also been isolated from breweries, wineries and mammalian sources. Of the six species present in this genus, the most important are *K. lactis* and *K. marxianus* (anamorph *C. kefyr*) for their capacity to ferment lactose.

2.4.1. *Taxonomic unit defined*

The investigation of the phylogenetic relationships among the members of the genus *Kluyveromyces* has revealed the existence of a monophyletic group that is the new *Kluyveromyces* genus, based on multigene sequences analysis (Kurtzman 2003). This genus is constituted by the species *K. aestuarii*, *K. dobzhanskii*, *K. lactis*, *K. marxianus*, *K. nonfermentans* and *K. wickerhamii*.

The best targets for identifying species of this genus are the sequences D1/ D2 (26S) or PCR and restriction of 5.8S-ITS.

The partial genome sequences are available for the following *Kluyveromyces* species:
- *K. lactis* (http://cbi.labri.fr/Genolevures/elt/KLLA)
- *K. marxianus* (http://cbi.labri.fr/Genolevures/about/GL1_genome.php)

2.4.2. Is the body of knowledge sufficient?

Humans. This microorganism can be isolated from milk products and is used as a starter to set up the medium for cheese and kefir production. *Kluyveromyces marxianus* and *K. lactis* are associated with

smear-ripened cheeses and contribute to the aromas that cheeses develop. These species are considered to be generally regarded as safe organisms and have been approved as a food additive (Coenen, Bertens *et al.* 2000).

Livestock. *Kluyveromyces* is used in animal feeds in Europe as a probiotic and is apparently safe (reviewed in Anadon, Martinez-Larranaga *et al.* 2006).

3.4.3. Are there safety concerns?

Candida kefyr, the anamorph of *K. marxianus*, has occasionally been involved in opportunistic infections in immunocompromised persons. However, considering the history of apparent safe use and the rarity of infections in humans, there are no safety concerns.

3.4.5. List of units proposed for QPS status

It is proposed to grant *K. lactis* and *K. marxianus* QPS status.

3.5. *Pichia*

Yeasts of the genus *Pichia* are widely distributed; they can be found in natural habitats, such as soil, freshwater, tree exudates, insects, plants and fruits, and also as contaminants in a variety of foods and beverages, including juices and soft drinks, alcoholic beverages, high sugar containing products, vegetables, meat and fermented products. Moreover, some *Pichia* species have also been found exhibiting desired effects in food, *e.g.* contributing in the early stages of wine fermentation, several types of brines, and different types of cheeses; while others have been described as human pathogens (Bakir, Cerikcioglu *et al.* 2004; Otag, Kuyucu *et al.* 2005).

3.5.1. Taxonomic unit defined

The genus *Pichia* is one of the largest yeast genera in view of the number of species. Since the genus was described in 1904, the number of species included in this taxon has changed considerably. *Pichia* currently contains 91 species with 30 being related to food production and processing. However, the majority of them can be considered as food contaminants (spoilage organisms). *Pichia* appears to be extremely heterogeneous. The genus nearly doubled in size with the transfer of nitrate-positive *Hansenula* species to *Pichia*. For example the previously-named *Hansenula polymorpha* and *H. angusta* are now know as *P. angusta*, and *H. anomala* is now called *P. anomala.*

The best targets for identifying species of this genus are the sequences D1/ D2 (26S) or PCR and restriction of 5.8S-ITS.

The genome sequence of *P. stipitis* (http://genome.jgi-psf.org/cgi-bin/browserLoad/457559ad1ccd245f58d7b393) and partial genome sequences of *P. angusta* (http://cbi.labri.fr/Genolevures/about/GL1_genome.php) are available.

3.5.2. Is the body of knowledge sufficient?

Humans. The genus contains the species previously encompassed in the genus *Hansenula*, which is reported to be one of the safest microorganisms; it is used by the WHO for the development of vaccines and as a producer organism (*e.g.* phytases). The main species are *P. anomala* (previously *Hansenula anomala*) *and P. angusta* (previously *Hansenula polymorpha*). *P. anomala* is also used for the fermentation of bakery products, while *P. roqueforti* is used as a post-harvest biocontrol agent for wheat and barley, or for food application (olive fermentations).

Pichia pastoris is frequently used as an expression system for the production of proteins. A number of properties makes *Pichia* suited for this task: *Pichia* has a high growth rate and is able to grow on a simple, inexpensive medium. *Pichia* can grow in either shake flasks or a fermentor, which makes it suitable for both small and large scale production. *Pichia pastoris* has a strong inducible promoter. This inducible promoter is related to the fact that *Pichia pastoris* is a methylotrophic yeast (Cereghino and Cregg 2000).

Pichia jadinii (anamorph *Candida utilis*), commonly called Torula, in its inactive form (usually labelled as Torula yeast), is widely used as a flavouring in processed foods and pet foods. It is produced from wood sugars, as a by-product of paper production. It is pasteurized and spray-dried to produce a fine, light greyish-brown powder with a slightly yeasty odour and gentle, slightly meaty taste.

Livestock. Some species of *Pichia* are used for feed (source of proteins), and as producer organisms (production of glucan for feed applications).

3.5.3. Are there safety concerns?

In the literature, *P. anomala* is described as a safe producer organism, since this yeast does not contain pyrogens or "viral inclusions" and is not a pathogen (Gellissen 2000).

However, there is a single report of *P. angusta* and *P. anomala* being responsible for cases of fungaemia in a Brazilian paediatric intensive care unit (Pasqualotto, Sukiennik *et al.* 2005). The source of the infection was never found. Patients with *P. anomala* fungaemia seem to have risk factors in common with those who have candidaemia. A number of transient cases of candidaemia caused by *Candida utilis* (*Pichia jadinii*) have been reported.

3.5.4. Can the safety concerns be excluded?

Considering the history of safe use and the rarity of infections in humans, there are no safety concerns.

3.5.5. List of units proposed for QPS status

It is proposed that *P. angusta* , *P. anomala, P. jadinii* and *P. pastoris* have QPS status.

3.6. *Saccharomyces*

These species are strongly fermentative, and are commonly isolated from soil, fruits, foods and beverages. *S. cerevisiae*, *S. pastorianus* and *S. bayanus* are widely used for making bread and in the production of beer, wine, distilled beverages and fuel alcohol. *S. cerevisiae* occurs on fruit, in processed fruits, dairy products and plays a role in the fermentation of kefir, coffee, cocoa, and the production of traditional fermented products. *S. cerevisiae* and *S. bayanus* cause spoilage of soft drinks.

3.6.1. Taxonomic unit defined

According to (Kurtzman 1999), the genus *Saccharomyces* includes 14 species. However more recently, and based on multigene sequence analysis, (Kurtzman 2003) proposed a new *Saccharomyces* genus that includes only seven of the previous species (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. cariocanus*, *S. kudriavzevii*, *S. pastorianus* and *S. bayanus*), the rest of the previous species are in a new genus, namely *Kazachstania*. The nucleotide sequence of the genome of each species has been determined but there are no annotations related to safety. Genome sequences are available for:

- *S. cerevisiae* (http://mips.gsf.de/genre/proj/yeast/ and http://www.yeastgenome.org)
- *S. cerevisiae* strain YJM789 isolated from the lung of fungal infections (http://www.ncbi.nlm.nih.gov/sites/entrez NC_009688)
- *S. bayanus* (http://www.broad.mit.edu/annotation/fungi/comp_yeasts/ and http://cbi.labri.fr/Genolevures/about/GL1_genome.php - partial sequence)
- *S. mikatae* (http://www.broad.mit.edu/annotation/fungi/comp_yeasts/)
- *S. paradoxus* (http://www.broad.mit.edu/annotation/fungi/comp_yeasts/)

The best targets for identifying species of this genus are the sequences D1/ D2 (26S) or PCR and restriction of 5.8S-ITS.

3.6.2. Is the body of knowledge sufficient?

Humans. The genus contains the most industrially exploited species known to man. It also contains the organism of choice as a model system for eukaryotic cell biology. As described above, this genus is used in the production of different foods, bread, beer, wine, distilled beverages and fuel alcohol, in processed fruits, dairy products and it plays a role in the fermentation of kefir, coffee, cocoa, and the production of traditional fermented products. Products are also commercialised as active dried yeast preparations. A subtype of *S. cerevisiae* (*Saccharomyces boulardii*) has been used by the food industry for many years as a probiotic, for horses as a treatment against acute enterocolitis, for human health against ulcerative colitis, in combination with standard antibiotics against *Clostridium difficile* disease, and as a treatment against persistent diarrhoea in children. *S. cerevisiae* is also used as biocontrol agent against *Solanaceae* diseases (Czerucka and Rampal 2002).

Livestock. *Saccharomyces* is used in animal feeds in the European Union as a probiotic (reviewed in (Anadon, Martinez-Larranaga *et al.* 2006)).

3.6.3. Are there safety concerns?

Saccharomyces cerevisiae (also known as "baker's yeast" or "brewers yeast") is mostly considered to be an occasional digestive commensal. However, since the 1990's, there have been a growing number of reports about its implication as an aetiological agent of invasive infection in "fragile" populations. A particular feature of such infections is their association with a probiotic preparation of *S. cerevisiae* (subtype *S. boulardii*) for treatment of various diarrhoeal disorders (see below). The nature of *S. cerevisiae* (subtype *S. boulardii*) and its clinical applications are reviewed by (Buts and Bernasconi 2005).

In one review, 92 cases of *Saccharomyces* invasive infection were presented (Enache-Angoulvant and Hennequin 2005). Predisposing factors were similar to those of invasive candidosis, with intravascular and antibiotic therapy being the most frequent. Blood was the most frequent site of isolation (78% or 72 patients). *S. cerevisiae* (subtype *S. boulardii*) accounted for 51.3% (47 cases) of fungaemias and was exclusively isolated from blood. Special caution should be taken regarding the use of *S. cerevisiae* (subtype *S. boulardii*) preparations (Fleet and Roostita, 2006). There are number of recent reports and reviews regarding the safety of *S. cerevisiae* (subtype *S. boulardii)* preparations involved in:

- A case of *Saccharomyces cerevisiae* acquired fungaemia (Cassone, Serra *et al.* 2003; Graf and Gavazzi 2007). The authors concluded that probiotics should be used cautiously in certain high-risk populations.
- A review of the current literature reinforces the view that fungaemia and sepsis are rare complications of the administration of *S. cerevisiae* (subtype *S. boulardii*) in immunocompromised patients but confirms that the most important risk factor for *S.*

cerevisiae fungaemia is the use of probiotics (Herbrecht and Nivoix 2005; Munoz, Bouza *et al.* 2005). This raises the question of the risk-benefit ratio of these agents in critically ill or immunocompromised patients who are likely to develop an infection after exposure to high amounts of a microorganism with a low virulence.

The body of knowledge is considered as sufficient (long history of safe use) with only 92 cases of pathogenic cases involving *S. cerevisiae* reported in total (15 cases diagnosed before 1990); all patients had at least one condition facilitating the opportunistic development of *S. cerevisiae*. *S. bayanus* and *S. pastorianus* are used in wine and beer production. There are no foodborne infection issues for these species.

3.6.4. Can the safety concerns be excluded?

It is possible to propose some species of the genus for QPS status with the following qualification: *S. cerevisiae,* subtype *S. boulardii* should certainly be contraindicated for patients of fragile health, as well as for patients with a central venous catheter in place. It is recommended that a specific protocol concerning the use of probiotics needs to be formulated.

3.6.5. List of units proposed for QPS status

S. bayanus, *S. cerevisiae* and *S. pastorianus* (syn of *S. carlsbergensis*) are proposed for QPS status with the above qualification.

3.7. *Schizosaccharomyces*

Three species are included in this genus, *Sch. japonicus*, *Sch. octosporus* and *Sch. pombe*. The species are isolated from fruits and fruit juices, wines, tequila fermentation and high sugar concentration. The species are strong fermenters of sugars and have been used for the production of ethanol.

The species *Sch. pombe* is used as a phytase producer for animal feed; minimum safety precautions should be taken for the handling and storage. No infection issues have been reported.

The best targets for identifying species of this genus are the sequences D1/ D2 (26S) or PCR and restriction of 5.8S-ITS.

The genome sequence for *Sch. pombe* is available (http://www.genedb.org/genedb/pombe/)

3.7.1. List of units proposed for QPS status

Sch. pombe is proposed for QPS status

3.8. *Xanthophyllomyces.*

Phaffia rhodozyma was isolated by Herman Phaff in the 1960s, during his pioneering studies of yeast ecology. The species ferments D-glucose and occurs in slime fluxes of deciduous trees. Initially, the yeast was isolated from limited geographical regions, but isolates were subsequently obtained from Russia, Chile, Finland, and the United States. The biological diversity of the yeast is more extensive than originally envisioned by Phaff and his collaborators, and at least two species appear to exist, including the anamorph *Phaffia rhodozyma* and the teleomorph *Xanthophyllomyces dendrorhous*. The yeast has attracted considerable biotechnological interest because of its ability to synthesize the economically important carotenoid astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione) as its major pigment. This property has stimulated research on the biology of the yeast as well as development of the yeast as an industrial microorganism for astaxanthin production by fermentation. The pigment is an important dietary source for aquaculture and poultry industries, including salmonids, lobsters and the egg yolks of chickens and quail, in order to impart characteristic and desirable colours.

The best targets for identifying species of this genus are the sequences D1/ D2 (26S).

There are no literature reports to suggest that this species may be hazardous to human health.

3.8.1. List of units proposed for QPS status

Due to a long history of apparent safe use *Xanthophyllomyces dendrorhous* is proposed for QPS status

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APPENDIX D: Scientific report on the assessment of filamentous fungi

Filamentous fungi are flexible microorganisms that can show different properties depending on the external factors (substrate, growth conditions, biotic/abiotic conditions). A consequence is the ability of a microorganism to produce different types and quantity of secondary metabolites depending on the growth conditions. Mycotoxins are well-known secondary metabolites, and *Penicillium roqueforti,* for example, is safely used for cheese production but could start to produce a lot of mycotoxins if the substrate is changed, *e.g.* to rye bread. Regarding the QPS status, the ability of fungal species to produce toxic metabolites represents the greatest difficulties. Based on the assumption that each of the estimated 1.5 million fungal species (Hawksworth 1991) can produce at least two unique secondary metabolites, there may be as many as 3 millions unique fungal metabolites. Approximately 10% of the secondary metabolites listed up till now have been classified as mycotoxins. Thus, there are potentially up to 300,000 unique mycotoxins (CAST 2003). The number of fungal metabolites and mycotoxins still undiscovered is therefore quite large and the diversity of toxic mechanisms will be equally as great. There is, unfortunately, no standardised method to consider fungal metabolites and their toxicity such as effect-based bioassay methods. The regulation of metabolites and their possible interactions are therefore poorly understood. Just as for other types of microorganisms, the toxic effect of a fungus used for food production will only be detected in case of acute toxicity, but not if it shows long term (chronic) toxicity (*e.g.* carcinogenic properties). In addition, the number of validated analytical methods for mycotoxins and other fungal metabolites is low and even for those available, analytical quality assurance procedures are often lacking (van Egmond 2004).

In contrast to bacteria, the spread of antimicrobial resistance through filamentous fungi is not a concern. Attention should, however, be paid to fungi capable of producing antibiotics that are not initially present in food and that therefore might contribute to the emergence of populations of resistant bacteria (*e.g.* penicillin production by some fungal species).

A market-research study (Sunesen 2003) illustrates the difficulty of getting precise information on the identity of organisms used in food, and therefore to evaluate their safety.

Mycological methods to be used for identification of moulds:

Filamentous fungi are traditionally identified to genus level by phenotypic characters, such as morphological and cultural characteristics. Unfortunately, there is not one universal mycological textbook or reference compendium which is used for identification of moulds, which makes identification to genus level a highly subjective task. This is further complicated by the necessity to identify fungal strains to the species level as each species within a genus may have very different functional characters, *e.g.* mycotoxin profiles and physiological properties. Again, traditional methods like morphological and cultural characteristics are widely used but also profiles of secondary metabolites have been used within some genera. Phenotypic characteristics do vary according to growth conditions which makes it difficult to construct robust identification keys. No

identification key covers all species, so it is recommended seeking advice for identification procedures by contacting specialists in food, feed and industrial mycology – *e.g.* via the International Commission on Food Mycology (ICFM) (http://www.foodmycology.org), which can direct inquires to recommended specialists.

For filamentous fungi the use of molecular biology based methods is less developed than for bacteria and yeasts. On the other hand, in combination with phenotypic studies, numerous phylogenetic studies using gene sequences have changed the systematics within mycology and will play an increasing role in the future by changing our understanding of species delimitations and relationships. As a spin-out from molecular biology, some sequenced-based identification schemes have been developed (*e.g.* for *Trichoderma* – see below) along with various PCR detection systems. However, the latter systems often are intended for a limited number of species, at times only a minor part of a genus. The molecular methods developed so far are not based on the same gene(s) for different genera and need further improvement (Paterson 2006). Many recent phylogenetic studies and molecular detection systems are based on a Multi-Locus Sequence Typing (MLST) concept, where sequences from several genes are used simultaneously. Typical targets chosen for MLST typing are "housekeeping" genes, without which the host organism will be unable to function. Again, there will be differences among fungal genera regarding the loci used in MLST studies and advice should be obtained by consulting specialists – *e.g.* via the International Commission on Food Mycology.

1. *ASPERGILLUS*

This genus is among the best known filamentous fungi, as *Aspergillus* species are widely used for production of chemicals (*e.g.* citric acid), enzymes and for biotransformations. On the other hand, *Aspergillus* species are also known to be among the most toxic spoilers of food and feed, some species are even pathogenic to man and food producing animals. No recent monograph on *Aspergillus* and its teleomorphic states exists, which makes it complicated to give a clear picture of the current status within this genus where the systematics are currently changing rapidly. Specific reviews will be cited for the relevant species in the paragraphs that follow. For a general introduction to *Aspergillus* it may be useful to consult some general texts on food mycology (Pitt 1979; Samson 2004), as well as a recent update on concepts for species differentiation in *Aspergillus* (Samson 2006).

1.1. *Aspergillus* section *Nigri* (the black Aspergilli)

The taxonomy of the section *Nigri* (the black Aspergilli) is not fully resolved as the number of accepted species depends on the methodology used. So far there has not been complete agreement between morphological, chemical and molecular data, but some generally acceptance has been proposed (Schuster, Dunn-Coleman *et al.* 2002; Abarca, Accensi *et al.* 2004; Samson 2004); however species identification remains problematic. The section *Nigri* includes 16 species *A. niger, A. foetidus, A. tubingensis, A. aculeatus, A. brasiliensis, A. carbonarius, A. costaricaensis, A. ellipticus, A. heteromorphus, A. homomorphus, A. ibericus, A. japonicus, A. lacticoffeatus, A.*

piperis, A. sclerotiniger, and *A. vadensis;* however only the first four species listed will be evaluated for a possible QPS status as they have been used for food or feed purposes, including enzyme production.

1.2. *Aspergillus niger*

In general *Aspergillus niger* sensu lato has a long history of apparent safe use in biotechnology, *e.g.* for the production of chymosin and other enzymes or citric acid (Schuster, Dunn-Coleman *et al.* 2002; van Dijck, Selten *et al.* 2003). *A. niger* is not known to be used as food or feed in Europe, even though this species has been evaluated for use as a source of single-cell protein (Christias, Couvaraki *et al.* 1975; Hang 1976; Singh, Abidi *et al.* 1991; Oboh 2002). Were a strain of *A. niger* to be allowed in Europe, it would fall under the Novel Food Regulation (258/97/EC) and would thus require a risk assessment under that legislation.

The full nucleotide sequences of the genomes of three strains of *Aspergillus niger sensu stricto* have been determined and information are available at these web sites:
http://genome.jgi-psf.org/Aspni1/Aspni1.home.html
http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/index.php~main

It is well documented that some strains of this species produce the mycotoxin ochratoxin A (Abarca, Bragulat *et al.* 1994; Samson 2004; Serra, Cabanes *et al.* 2006). Other metabolites with poorly documented biological activity from *A. niger* are: pyranonigrin, kotanins and naphtho-γ-pyrones (Samson 2004). *A. niger* is the third most common species associated with invasive pulmonary aspergillosis and it is also often a causative agent of aspergilloma (Kwon-Chung 1992). It is also a recognised opportunistic pathogen for animals and there have been reports of natural aspergillosis in various species of mammals and birds (Smith 1989).

Despite the long history of apparent safe use in biotechnology, where strain improvement combined with cleaning and purification steps have been added to processes to eliminate metabolites other than the product of interest (van Dijck, Selten *et al.* 2003; Blumenthal 2004), industrial strains of *A. niger* have been proven to produce ochratoxin A (Schuster, Dunn-Coleman et al. 2002) which makes *A. niger* ineligible for a QPS status.

1.3. ***Aspergillus foetidus, Aspergillus tubingensis* and *Aspergillu aculeatus***

are each used for enzyme production. Even though *A. aculeatus* is from Section *Nigri* it can be distinguished morphologically from *A. niger* and the other species in the *Nigri* section (Pitt 1997). Nevertheless, due to the confused taxonomy of the Section *Nigri* in the past, many reports on enzyme production by *A. niger* should probably be attributed to isolates of *A. foetidus, A. tubingensis* or *A. aculeatus.* These species are known to produce many metabolites with poorly described biological activity. For *A. foetidus*, these include pyranonigrin, naphtho-γ-pyrones, asperazine, and anatafumicin (Samson 2004). *A. tubingensis* has been reported to produce pyranonigrin, naphtho-γ-pyrones, and asperazine, (Samson 2004), and *A. foetidus* to produce ochratoxin A (Teren, Varga *et al.* 1996;

Bragulat, Abarca *et al.* 2001; Abarca, Accensi *et al.* 2004). One metabolite from *A. aculeatus*, secalonic acid, is known to be a mycotoxin (Samson 2004).

Despite the long history of apparent safe use in biotechnology, the body of knowledge concerning the toxicological aspects of the metabolites is insufficient, which makes *Aspergillus foetidus, A. tubingensis* and *A. aculeatus* ineligible for a QPS status.

1.4. Other Aspergilli

1.4.1. *Aspergillus candidus*

A. candidus can be found in meat products (sausages) as a starter culture with a long history of traditional use with regard to the house mycobiota (Sunesen 2003). This species is not produced commercially as starter culture (for application by spraying or dipping), hence it is not declared. *A. candidus* can also be found as a contaminant (food spoiler) in cereals and many other food products (Pitt 1997).

Despite the frequent occurrence of this species the body of knowledge is considered as insufficient; it produces known metabolites, some of them showing cytotoxic activity: AcT1 (Chattopadhyay, Nandi *et al.* 1987), xanthoascin (Ito, Ohtsubo *et al.* 1978), terphenyllin (Marchelli and Vining 1975; Stead, Affleck *et al.* 1999). However, there remains metabolites that are not yet identified and classified (Samson 2004; Andersen and Thrane 2006). The toxicology of the metabolites of *A. candidus* is unknown, so the safety concerns cannot be excluded. Even if it is possible to get rid of most of the fungal biomass by washing the surface of the product, there is the possibility that fungal metabolites will remain on the product. Moreover, possible interactions between these metabolites have yet to be investigated. Some rare case of infections linked to *A. candidus* can be found in the literature (Kwon-Chung 1992; Ribeiro, Santana *et al.* 2005).

In conclusion, considering that *Aspergillus candidus* is known to produce secondary metabolites with poorly understood toxicity for which there is no data on possible interactions, and that *A. candidus* is mainly used for food production as a house starter culture and therefore mixed with other fungi, makes *A. candidus* ineligible for a QPS status.

1.4.2. *Aspergillus oryzae*

In Asia a long tradition of using fungal cultures to produce fermented food such as sake (rice wine), shoyu (soy sauce) and miso (soybean paste) exists. These products are fermented by "koji-moulds", which consist principally of *Aspergillus oryzae*, but may also contain *A. "awamori"* (=*A. niger*) , *A. sojae* and *A. tamarii*. The consumption of these fermented foods in Japan has been considered as safe (Tanaka 2006). Recent genomic approaches have demonstrated that *A. oryzae* and *A. tamarii* are taxonomically closely related to *A. flavus*, while *A. sojae* and *A. awamori* are genetically related to *A. parasiticus* and *A. niger*, respectively (Machida, Asai *et al.* 2005).

Aspergillus oryzae has a long history of apparent safe use, both in food outside Europe (it is one of the main species used in Asia for the production of soy sauce, which is exported worldwide), and for enzyme / protein production (cell factory), however, this is as GM organisms (Archer 2000). *A. oryzae* is accepted as a domesticated form of *A. flavus* (Pitt 1997), which is an aflatoxin producer. The phenotypic distinction between *A. oryzae* and *A. flavus* is difficult as only fine details in conidial ornamentation and colony characteristics (*i.e.* colour of conidial mass and colour of colony reverse on Aspergillus Flavus Parasiticus Agar) separate the two (Samson 2004). However, by several molecular methods it has not been possible to separate the two into distinct species (Cary and Ehrlich 2006; Chang, Ehrlich *et al.* 2006). *A. oryzae* has the gene cluster for aflatoxin but has a minute change in the sequence for a regulatory gene, *aflR*, which is believed to the reason for the absence of aflatoxin production by *A. oryzae* (Lee, Liou *et al.* 2006). A recent review of the occurrence of aflatoxins and their production by various koji-moulds (Tanaka 2006) demonstrated that 212 strains used for fermentation of different foods were negative for aflatoxin production. Aflatoxins were not detected in any of the 289 food samples analysed (rice, soy sauce, soybean paste).

Strains of *A. ozyzae* do, however, produce the mycotoxins cyclopiazonic acid, which is a neurotoxic and immunosuppressive compound, and β-nitropropionic acid and kojic acid (Samson 2004). Four of 36 *A. oryzae*-strains used commercially were found to be producers of cyclopiazonic acid (Goto 1987), whereas kojic acid was found to be produced by 85 of 149 koji-mould strains used commercially (Shinshi 1984). The strains producing toxin were removed from commercial use and Tanaka *et al.* (Tanaka 2006) concluded that the risk for mycotoxin contamination of typical Japanese fermented food can be classified as very low. *A. oryzae* is also used as feed for dairy cows and beef cattle in growth finishing stages; however, potential production of cyclopiazonic acid and β-nitropropionic acid were not taken into consideration (EFSA 2006).

Despite the long history of apparent safe use in food and biotechnology, where cleaning and purification steps have been added in the process to get rid of all metabolites but the product of interest (Blumenthal 2004), the body of knowledge concerning the formation of well-known mycotoxins, cyclopiazonic acid and β-nitropropionic acid, under production conditions as well as any long-term toxicological aspects of these toxins is insufficient. In addition, no universally accepted method for an unambiguous identification of *A. oryzae* exists, which make *Aspergillus oryzae* not suitable for a QPS status.

2. *PENICILLIUM*

Among the most frequently encountered fungi in food and feed systems are species of the genus *Penicillium,* which are very well-known as spoilers and mycotoxin producers but also as starter cultures for products like *e.g.* white- and blue-mould cheeses and mould-ripened meat products. The modern systematics of the genus *Penicillium* was initiated by a monograph more than 25 years ago (Pitt 1979) and has developed dramatically since then. Today the genus is divided into four subgenera (Pitt 1997; Samson 2004) and may contain more than 500 species. Many species, however, are soil fungi and has never been related to food and feed systems, except as occasional spoilers. All *Penicillium* species are good producers of mycotoxins and other biological active

metabolites, however the available literature is overwhelming and difficult to interpret as the identification of *Penicillium* cultures is not trivial and has resulted in numerous misidentifications (Frisvad, Nielsen *et al.* 2006). Partly as a consequence of this, starter cultures are often vaguely labelled as "*Penicillium* spores" (Sunesen 2003). Among the *Penicillium* strains used routinely in the food industry toxigenic strains are frequent. In a study of 249 *Penicillium* strains originally isolated from food products and used as starter cultures only 13 isolates were found to meet the demands on technological suitability and toxicological safety, which includes the testing of the strains with regard to the production of antibiotic, cytotoxic and mutagenic metabolites (Gareis 1999).

Based on literature reviews, only species within the subgenus *Penicillium* have been used as starter cultures for food and feed. Recently this subgenus has been the subject of a monograph (Frisvad 2004; Samson 2004; Smedsgaard 2004) including an extensive review on the related secondary metabolites (Frisvad 2004). An interactive identification key based on phenotypic characters and β-tubulin gene sequences is available at:
http://www.cbs.knaw.nl/penicillium/DefaultPage.aspx.

2.1. *Penicillium camemberti*

Penicillium camemberti has a long history of use in cheese production (camembert cheese and white mould cheeses in general) often declared by the use of invalid synonyms as *P. album, P. candidum*, *P. casei* or *P. caseicola*. It is also found as a spontaneous coloniser on fermented sausages originating from the local mycobiota of the production plant (Sunesen 2003) and as a starter culture to give aroma to fermented meat products. This species is also used for enzyme production (Pariza and Johnson 2001). The taxonomy of *P. camemberti* is well known and this species is accepted as a domesticated form of *P. commune.*

There are no reports of an adverse health effect for cheese or meat produced with *P. camemberti*, *i.e.* no acute toxicity associated with food produced by *P. camemberti* has been reported. This species is however known as a producer of cyclopiazonic acid (CPA), this being a neurotoxic and immunosuppressive compound (Frisvad 2004); unknown cytotoxic metabolites are also produced when this fungus is used as a starter culture for mould-ripened meat products (Gareis 1999). A few strains also produce metabolites with poorly described biological activity, such as cyclopaldic acid, rugulovasine A & B and palitantin (Frisvad 2004). CPA has been detected in cheeses at 0.25-0.37 mg/kg cited by (Pitt 1997) and in meat products cited by (Sunesen 2003). Naturally occurring mutants that do not produce this mycotoxin have been reported (Geisen, Glenn *et al.* 1990). There are not enough toxicological data available to set a threshold under which the consumption of cyclopiazonic acid does not pose any risk. It is important to note that *P. camemberti* (mostly cited as *P. casei*) is known as the aetiological agent of the "cheese worker's lung" associated with hypersensitivity pneumonitis (Campbell, Kryda *et al.* 1983; Marcer, Franchini *et al.* 1996).

Despite the long history of apparent safe use of *P. camemberti,* the capacity of this microorganism to produce cyclopiazonic acid, even under known production conditions, makes *P. camemberti* ineligible for QPS status.

2.2. *Penicillium chrysogenum*

Penicillium chrysogenum is used as a starter culture for the production of dry sausages (Sunesen 2003) and is also used in the pharmaceutical industry to produce penicillin. It is also known to produce roquefortine C, PR-toxin and secalonic acids, which are mycotoxins, in addition to secondary metabolites with poorly described biological activity: chrysogine, xanthocillin, sorrentanone and sorbicillin (Frisvad 2004).

Considering the capacity of this species to produce unwanted antibiotics in food, each strain should be investigated in detail, which makes *P. chrysogenum* ineligible for QPS status.

2.3. *Penicillium funiculosum*

P. funiculosum is used as a producer of enzyme preparation intended for animal feed (Das and Singh 2004) and also as a host for the production of heterologous proteins. The promoter of the histone H4.1 gene was successfully used to drive the expression of an intracellular bacterial enzyme, β-glucuronidase, and a secreted homologous enzyme, xylanase C (Belshaw, Haigh *et al.* 2002). In general, no known mycotoxins are known from this species; however a single strain has been shown to produce the mycotoxin secalonic acid (JC Frisvad, pers. comm.). Cultures of *P. funiculosum* do produce many secondary metabolites of unknown structure and unknown biological activity, hence *P. funiculosum* is ineligible for QPS status.

2.4. *Penicillium nalgiovense*

Penicillium nalgiovense is widely used as starter culture for the production of dry sausages (Sunesen 2003). Wild-type isolates from meats and cheeses have green conidia, whereas starter cultures have white conidia. This species produces penicillin and a broad range of secondary metabolites with poorly described biological activity: nalgiovensin, nalgiolaxin, diaporthins and dipodazin (Frisvad 2004). Typically isolates from meats are good producers of penicillin, while cheese isolates produce penicillin in low amounts. In addition, some strains have been found to produce cytotoxic metabolites on nutrient agar and mould-ripened salamis (Gareis 1999).

Despite the long history of apparent safe use of *P. nalgiovense,* the capacity of this species to produce unwanted antibiotics and cytotoxic metabolites in food makes *P. nalgiovense* ineligible for QPS status.

2.5. *Penicillium roqueforti*

Penicillium roqueforti has a long history of apparent safe use in the production of blue-moulded cheeses, but is also often isolated from rye bread, silage and other acid preserved products. *P. roqueforti* has also been reported as a source for enzymes used in food processing (Pariza and Johnson 2001).

Ten years ago, two closely related species, *P. paneum* and *P. carneum*, were discovered (Boysen, Skouboe *et al.* 1996). All three share many ecological and morphological features, which makes it difficult to interpret older literature, however their profiles of secondary metabolites are distinct (Frisvad 2004; Nielsen 2006).

P. roqueforti sensu stricto produces the mycotoxins roquefortine C & D, PR-toxin, mycophenolic acid, isofumigaclavine A & B and metabolites with poorly described biological activity: citreoisocoumarin and α-amino butyric acid peptides (peptaibols) (Frisvad 2004). The related species *P. carneum* produces the mycotoxins mycophenolic acid, patulin, roquefortine C, penitrem A, isofumigaclavine A, as well as cyclopaldic acid with a poorly described activity. *P. paneum* produces the mycotoxins patulin, roquefortine C & D, botryodiploidin and metabolites with poorly described biological activity: marcfortines and citreoisocoumarin (Frisvad 2004). For *P. roqueforti sensu stricto* roquefortine and PR-toxin productions are occurring in cheese but at amounts that are not considered as toxic for humans (Pitt 1997). There is no data on possible long-term toxic effects. In general toxicological data for *P. roqueforti* metabolites are insufficient to set a threshold for regulatory purposes.

Despite the long history of apparent safe use of *P. roqueforti*, this species is ineligible for QPS status as no validated analytical methods for the mycotoxins exist to qualify for the absence of mycotoxins under production conditions.

3. *TRICHODERMA*

There have been many developments within the taxonomy and systematics of this genus lasting recent years (Druzhinina and Kubicek 2005; Samuels 2006) and interactive identification key to the more than 90 species of *Trichoderma* and its teleomorph, *Hypocrea* has been developed based on molecular methods (Druzhinina, Kopchinskiy *et al.* 2005; Kopchinskiy, Komon *et al.* 2005) located at http://www.isth.info/index.php
Another interactive key based on morphological and cultural characters for identification of *Trichoderma* and some of its teleomorphs is also available and has many illustrations. This key is located at http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm

The available literature on bioactive compounds from *Trichoderma* species is extensive and was reviewed some years ago (Sivasithamparam 1998). Since then, numerous reports have published, however no production of compounds classified as mycotoxins have been reported. For many years the production of trichothecene mycotoxins have been associated with several *Trichoderma* species, but it has now been clarified that the trichothecene producing species is a newly described species, *T. brevicompactum*, and not any of those species listed below (Nielsen, Grafenhan *et al.* 2005).

Trichoderma species are known to be aggressive and are used as biocontrol agents, however the difficult systematics is a challenge when it comes to identifying exactly which species is involved (Kullnig 2001; Hermosa, Keck *et al.* 2004). An EU sponsored initiative to evaluate biological control agents, REBECA, has been launched – see http://www.rebeca-net.de for details.

3.1. *Trichoderma reesei*

Trichoderma reesei is widely used for enzyme production and the toxicological evaluations that need to be taken into consideration have been reported (Blumenthal 2004). However, the potential production of trichothecenes can be neglected as this species cannot produce these mycotoxins (Nielsen, Grafenhan *et al.* 2005). *T. reesei* is reported to produce peptaibol compounds which are known to disintegrate cell membranes, causing therefore apoptosis (Bruckner and Graf 1983), as well as other biological active cyclopeptides (Sun, Tian *et al.* 2006).

Considering the capacity of this species to produce unwanted biological active compounds, each strain should be investigated in detail, which makes *T. reesei* ineligible for QPS status.

3.2. *Trichoderma harzianum*

Trichoderma harzianum is mainly used as a bio-control agent (Harman, Howell *et al.* 2004) and some strains are known to be very aggressive to (plant pathogenic) mushrooms (Samuels 2002). *T. harzianum* is known to produce a high number of secondary metabolites with partly characterised biological activity (Sivasithamparam 1998; Hanson 2005); however it is known that 6-n-pentyl-α-pyrone (coconut smell) is responsible for at least part of the biological aggressiveness of this species and that highly biologically active α-amino butyric acid cyclic peptides (peptaibols) are involved in the apoptosis mechanism, in addition to anthraquinones, azaphilones, harzianolide and harzianopyrione which have different activities towards plant pathogens (Vinale, Marra *et al.* 2006).

Considering the capacity of this species to produce unwanted biological active compounds, each strain should be investigated in detail, which makes *T. harzianum* ineligible for QPS status.

3.3. *Trichoderma viride*

Trichoderma viride has been evaluated for single cell production (Hang 1976; Youssef 1999), but this has never been commercialised. This species is not used as a bio-control agent but is considered very aggressive and has been reported to produce 6-n-pentyl-α-pyrone (coconut smell) and several biologically active α-amino butyric acid cyclic peptides (peptaibols) in addition to many secondary metabolites with poorly described biological activity (Sivasithamparam 1998). Possibly many production strains are misidentified according to an updated taxonomy; however this cannot be proven as many strains are no longer available. *T. viride* has been associated with human fungal infections (De Miguel, Gomez *et al.* 2005).

Considering the capacity of this species to produce many biological active compounds, each strain should be investigated in detail, which makes *T. viride* ineligible for QPS status.

3.4. *Trichoderma longibrachiatum*

Trichoderma longibrachiatum has been reported as a potential bio-control agent (Kullnig 2001; Vizcaino, Sanz *et al.* 2005). This species is considered very aggressive and has been reported to produce several biologically active α-amino butyric acid cyclic peptides (peptaibols) (Mohamed-Benkada, Montagu *et al.* 2006) in addition to many secondary metabolites with poorly described biological activity (Sivasithamparam 1998; Sperry 1998; Vicente, Cabello *et al.* 2001). Possibly many production strains are misidentified according to an updated taxonomy; however this cannot be proven as many strains are no longer available. *T. longibrachiatum* has been associated with from human fungal infections (Kuhls, Lieckfeldt *et al.* 1999; De Miguel, Gomez *et al.* 2005).

Considering the capacity of this species to produce many biological active compounds, each strain should be investigated in detail, which makes *T. longibrachiatum* ineligible for QPS status.

4. *FUSARIUM*

Currently, the genus *Fusarium* contains about 150 species; however the systematics are now changing rapidly due to the rapid developments in molecular biology. Many recently-described *Fusarium* species have been discovered by molecular tools used in phylogenetic studies, followed by a formal description of the species (Skovgaard 2003; O'Donnell, Ward *et al.* 2004; Aoki 2005). Introductions to *Fusarium* are available (Leslie 2001; Summerell 2003; Samson 2004; Leslie 2006) along with extended information on the mycotoxin production by *Fusarium* species (Marasas 1984; Thrane 2001; Sewram, Mshicileli *et al.* 2005; Andersen and Thrane 2006). Only one species is used in food production.

4.1. *Fusarium venenatum*

The only commercial mycoprotein products for human food are based on *Fusarium venenatum* biomass (Quorn® products from Marlow Foods Ltd.). The biotechnological development of these products is well described (Wiebe 2002). The major concern is that *F. venenatum* is a potential producer of mycotoxins, such as trichothecenes (diacetoxyscirpenol [DAS]) and several derivatives thereof, nivalenol and fusarenon X), butenolide and culmorin (Thrane and Hansen 1995; Miller 2000; Nielsen and Thrane 2001), which are carefully controlled and monitored during mycoprotein production (Johnstone 1998). Strains of *F. venenatum* which are used to produce enzymes are genetically modified (Royer, Moyer *et al.* 1995; Royer, Christianson *et al.* 1999; Pedersen and Broadmeadow 2000; Ahmad, Brinch *et al.* 2004).

Considering that *F. venenatum* is very toxic as wild-type, and that all strains used for enzyme production are genetically modified, this species is ineligible for QPS status.

5. *MONASCUS*

Monascus species (*M. purpureus, M. ruber, M.* spp) are known to produce yellow, orange and red pigments. Traditionally, *Monascus* has been cultured on rice and other cereals by solid sate fermentation. The red-coloured rice (Anka or Ang-kak) has been used for centuries in Asia as natural food colorant for bean curd, meat, wine and other foods. Nowadays, the purified pigments are widely used as colorants in processed seafood, sausages and sauce in Asia. In addition, extracts and other red-mould rice preparations are sold through the internet as nutritional additives with claims that they will lower blood cholesterol levels. No direct adverse health aspects have been reported. However, several studies have shown the presence of the mycotoxin citrinin, which is nephrotoxic and therefore an undesirable toxic secondary metabolite, among the pigments of *Monascus* and in commercial *Monascus*-preparations (Blanc, Laussac *et al.* 1995; Dietrich 1999; Xu 1999). The allergenic relevance of *M. purpureus* was the first time shown in 2002 (Hipler, Wigger-Alberti *et al.* 2002).

The European Community legislation on food additives is based on the principle that only those additives that are explicitly authorised may be used. Pigments from *Monascus* and *Monascus* preparations are not included in the list of permitted food colours of the European Parliament and Council Directive. As no toxicological safe and technologically efficient strains of *Monascus* are available for general use, no species within this genus is eligible for QPS status.

6. *RHIZOMUCOR*

Rhizomucor miehei and *Rh. pusillus* are the valid names for the thermophilic fungi *Mucor miehei* and *M. pusillus*, respectively (Schipper 1978) and both are used to produce chymosin, dextranase and protease with rennet-like activity (Pariza and Johnson 2001). Extensive literature searches have not retrieved any information on toxic compounds produced by these two species. In addition, the WHO has evaluated enzymatic preparations from *Rh. miehei* and *Rh. pusillus* and concluded that no adverse effects could be observed (WHO 1975; WHO 1975).

Despite the apparent safe use as an enzyme producing organism, it has not been possible through extensive literature searches to verify a general absence of biological active secondary metabolites, including allergenic compounds, from *Rhizomucor* species. Thus, species from this genus cannot be proposed for QPS status.

7. *CRYPHONECTRIA PARASITICA (SYN. ENDOTHIA PARASITICA)*

Cryphonetria parasitica is the valid name for *Endothia parasitica* (Barr 1978) and this fungus is used to produce protease with rennet-like activity (Pariza and Johnson 2001). WHO has evaluated enzymatic preparations from *Cr. parasitica* and concluded that no adverse effects could be observed (WHO 1975). However, this species have been reported to produce rugulosin and skyrin (Frisvad 1993). These compounds with poorly described biological activity have also been found in the fermentation batches.

Despite the long history of apparent safe use of enzyme production by *Cryphonetria parasitica,* the capacity of this microorganism to produce biological active compounds under production conditions makes it ineligible for QPS status.

8. *BLAKESLEA TRISPORA*

Blakeslea trispora is used to produce carotenoids in well established commercial products; these naturally produced food colorants are usually not purified. An extensive literature search did not reveal any information on toxic metabolites from this species. In addition, the AFC Panel of EFSA concluded that the toxicity data on lycopene from *B. trispora* is not of concern as long as the mean intake of lycopene from coloured food does not exceed the intake from natural sources (EFSA 2005).

Despite the apparent safe use as a colorant producing organism, it has not been possible through extensive literature searches to verify a general absence of biological active secondary metabolites, including allergenic compounds, from *Blakeslea trispora*. Thus, this species cannot be proposed for QPS status.

CONCLUSION

No filamentous fungi can be proposed for a QPS status. The rationale for this is that the methods for identification of fungal cultures to genus/species level are very difficult and often need mycological expertise. There is an ongoing debate on species concepts in the mycological society which result in a lack of a universally accepted fungal taxonomy. This makes identification of fungal cultures intended for commercial use not a trivial issue and often the result should be verified by one or more independent specialists. For the time being there is no universally accepted method for fungal identification.

The body of knowledge concerning production of toxic compounds is insufficient, as far too little is known about the factors controlling the production of these compounds. In several cases it has been demonstrated that toxic compounds can be produced under production conditions, but often this information is not available. In addition, there are only few validated and certified analytical methods for the detection of a limited number of mycotoxins. For the majority of fungal secondary metabolites no validated method exists.

The body of knowledge concerning the toxicology of fungal secondary metabolites is insufficient. Bioassays are developed to address specific needs and are not validated. Often the toxicological knowledge is of little or no relevance to real life situations, *e.g.* lack of information on synergistic effects. The long history of use is not equal to safety, as many fungal metabolites are known to affect the immune system, which could lead to secondary infections. Also the knowledge on long-term effects is insufficient. In conclusion, all fungal species and strains notified to EFSA should be evaluated on a case-by-case basis.

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may be made available to the public without further notice.

[60 FR 16332, Mar. 29, 1995, as amended at 62 FR 17932, April 11, 1997]

PART 725—REPORTING REQUIREMENTS AND REVIEW PROCESSES FOR MICROORGANISMS

Subpart A—General Provisions and Applicability

Subpart B—Administrative Procedures

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Subpart L—Additional Procedures for Reporting on Significant New Uses of Microorganisms

Subpart M—Significant New Uses for Specific Microorganisms

AUTHORITY: 15 U.S.C. 2604, 2607, 2613, and 2625.

SOURCE: 62 FR 17932, April 11, 1997, unless otherwise noted.

Subpart A—General Provisions and Applicability

§ 725.1 Scope and purpose.

(a) This part establishes all reporting requirements under section 5 of TSCA for manufacturers, importers, and processors of microorganisms subject to TSCA jurisdiction for commercial purposes, including research and development for commercial purposes. New microorganisms for which manufacturers and importers are required to report under section 5(a)(1)(A) of TSCA are those that are intergeneric. In addition, under section 5(a)(1)(B) of TSCA, manufacturers, importers, and processors may be required to report for any microorganism that EPA determines by rule is being manufactured, imported, or processed for a significant new use.

(b) Any manufacturer, importer, or processor required to report under section 5 of TSCA (see § 725.100 for new microorganisms and § 725.900 for significant new uses) must file a Microbial Commercial Activity Notice (MCAN) with EPA, unless the activity is eligible for a specific exemption as described in this part. The general procedures for filing MCANs are described in subpart D of this part. The exemptions from the requirement to file a MCAN are for certain kinds of contained activities (see §§ 725.424 and 725.428), test marketing activities (see § 725.300), and research and development activities described in paragraph (c) of this section.

(c) Any manufacturer, importer, or processor required to file a MCAN for research and development (R&D) activities may instead file a TSCA Experimental Release Application (TERA) for a specific test (see § 725.250). A TERA is not required for certain R&D activities; however a TERA exemption does not extend beyond the research and development stage, to general commercial use of the microorganism, for which compliance with MCAN requirements is required. The TERA exemptions are for R&D activities subject to other Federal agencies or programs (see § 725.232), certain kinds of contained R&D activities (see § 725.234), and R&D activities using certain listed microorganisms (see § 725.238).

(d) New microorganisms will be added to the Inventory established under section 8 of TSCA once a MCAN has been received, the MCAN review period has expired, and EPA receives a Notice of Commencement (NOC) indicating that manufacture or importation has actually begun. New microorganisms approved for use under a TERA will not be added to the Inventory until a MCAN has been received, the MCAN review period has expired, and EPA has received an NOC.

§ 725.3 Definitions.

Definitions in section 3 of the Act (15 U.S.C. 2602), as well as definitions contained in §§ 704.3, 720.3, and 721.3 of this chapter, apply to this part unless otherwise specified in this section. In addition, the following definitions apply to this part:

Consolidated microbial commercial activity notice or *consolidated MCAN* means any MCAN submitted to EPA that covers more than one microorganism (each being assigned a separate MCAN number by EPA) as a result of a prenotice agreement with EPA.

Containment and/or inactivation controls means any combination of engineering, mechanical, procedural, or biological controls designed and operated to restrict environmental release of viable microorganisms from a structure.

Director means the Director of the EPA Office of Pollution Prevention and Toxics.

Exemption request means any application submitted to EPA under subparts E, F, or G of this part.

General commercial use means use for commercial purposes other than research and development.

Genome means the sum total of chromosomal and extrachromosomal genetic material of an isolate and any descendants derived under pure culture conditions from that isolate.

Health and safety study of a microorganism or *health and safety study* means any study of any effect of a microorganism or microbial mixture on health or the environment or on both, including underlying data and epidemiological studies, studies of occupational exposure to a microorganism or microbial mixture, toxicological, clinical, and ecological, or other studies of a microorganism or microbial mixture, and any test performed under the Act. Microorganism identity is always part of a health and safety study of a microorganism.

(1) It is intended that the term "health and safety study of a microorganism" be interpreted broadly. Not only is information which arises as a result of a formal, disciplined study included, but other information relating to the effects of a microorganism or microbial mixture on health or the environment is also included. Any data that bear on the effects of a microorganism on health or the environment would be included.

(2) Examples include:

(i) Tests for ecological or other environmental effects on invertebrates, fish, or other animals, and plants, including: Acute toxicity tests, chronic toxicity tests, critical life stage tests, behavioral tests, algal growth tests, seed germination tests, plant growth or damage tests, microbial function tests, bioconcentration or bioaccumulation tests, and model ecosystem (microcosm) studies.

(ii) Long- and short-term tests of mutagenicity, carcinogenicity, or teratogenicity; dermatoxicity; cumulative, additive, and synergistic effects; and acute, subchronic, and chronic effects.

(iii) Assessments of human and environmental exposure, including workplace exposure, and impacts of a particular microorganism or microbial mixture on the environment, including surveys, tests, and studies of: Survival and transport in air, water, and soil; ability to exchange genetic material with other microorganisms, ability to colonize human or animal guts, and ability to colonize plants.

(iv) Monitoring data, when they have been aggregated and analyzed to measure the exposure of humans or the environment to a microorganism.

(v) Any assessments of risk to health and the environment resulting from the manufacture, processing, distribution in commerce, use, or disposal of the microorganism.

Inactivation means that living microorganisms are rendered nonviable.

Institutional Biosafety Committee means the committees described in the NIH Guidelines in section IV.B.2.

Intergeneric microorganism means a microorganism that is formed by the deliberate combination of genetic material originally isolated from organisms of different taxonomic genera.

(1) The term "intergeneric microorganism" includes a microorganism which contains a mobile genetic element which was first identified in a microorganism in a genus different from the recipient microorganism.

(2) The term "intergeneric microorganism" does not include a microorganism which contains introduced genetic material consisting of only well-characterized, non-coding regulatory regions from another genus.

Introduced genetic material means genetic material that is added to, and remains as a component of, the genome of the recipient.

Manufacture, import, or process for commercial purposes means:

(1) To import, produce, manufacture, or process with the purpose of obtaining an immediate or eventual commercial advantage for the manufacturer, importer, or processor, and includes, among other things, "manufacture" or "processing" of any amount of a microorganism or microbial mixture:

(i) For commercial distribution, including for test marketing.

(ii) For use by the manufacturer, including use for product research and development or as an intermediate.

(2) The term also applies to substances that are produced coincidentally during the manufacture, processing, use, or disposal of another microorganism or microbial mixture, including byproducts that are separated from that other microorganism or microbial mixture and impurities that remain in that microorganism or microbial mixture. Byproducts and impurities without separate commercial value are nonetheless produced for the purpose of obtaining a commercial advantage, since they are part of the manufacture or processing of a microorganism for commercial purposes.

Microbial commercial activity notice or *MCAN* means a notice for microorganisms submitted to EPA pursuant to section 5(a)(1) of the Act in accordance with subpart D of this part.

Microbial mixture means any combination of microorganisms or microorganisms and other chemical substances, if the combination does not occur in nature and is not an article.

Microorganism means an organism classified, using the 5-kingdom classification system of Whittacker, in the kingdoms Monera (or Procaryotae), Protista, Fungi, and the Chlorophyta and the Rhodophyta of the Plantae, and a virus or virus-like particle.

Mobile genetic element or *MGE* means an element of genetic material that has the ability to move genetic material within and between organisms. "Mobile genetic elements" include all plasmids, viruses, transposons, insertion sequences, and other classes of elements with these general properties.

New microorganism means a microorganism not included on the Inventory.

NIH Guidelines means the National Institutes of Health (NIH) "Guidelines for Research Involving Recombinant DNA Molecules" (July 5, 1994).

Non-coding regulatory region means a segment of introduced genetic material for which:

(1) The regulatory region and any inserted flanking nucleotides do not code for protein, peptide, or functional ribonucleic acid molecules.

(2) The regulatory region solely controls the activity of other regions that code for protein or peptide molecules or act as recognition sites for the initiation of nucleic acid or protein synthesis.

Small quantities solely for research and development (or "small quantities solely for purposes of scientific experimentation or analysis or research on, or analysis of, such substance or another substance, including such research or analysis for development of a product") means quantities of a microorganism manufactured, imported, or processed or proposed to be manufactured, imported, or processed solely for research and development that meet the requirements of § 725.234.

Structure means a building or vessel which effectively surrounds and encloses the microorganism and includes features designed to restrict the microorganism from leaving.

Submission means any MCAN or exemption request submitted to EPA under this part.

Technically qualified individual means a person or persons:

(1) Who, because of education, training, or experience, or a combination of these factors, is capable of understanding the health and environmental risks associated with the microorganism which is used under his or her supervision,

(2) Who is responsible for enforcing appropriate methods of conducting scientific experimentation, analysis, or microbiological research to minimize such risks, and

(3) Who is responsible for the safety assessments and clearances related to the procurement, storage, use, and disposal of the microorganism as may be appropriate or required within the scope of conducting a research and development activity.

TSCA Experimental Release Application or *TERA* means an exemption request

for a research and development activity, which is not eligible for a full exemption from reporting under § 725.232, 725.234, or 725.238, submitted to EPA in accordance with subpart E of this part.

Well-characterized for introduced genetic material means that the following have been determined:

(1) The function of all of the products expressed from the structural gene(s).

(2) The function of sequences that participate in the regulation of expression of the structural gene(s).

(3) The presence or absence of associated nucleotide sequences and their associated functions, where associated nucleotide sequences are those sequences needed to move genetic material including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites.

§ 725.8 Coverage of this part.

(a) *Microorganisms subject to this part.* Only microorganisms which are manufactured, imported, or processed for commercial purposes, as defined in § 725.3, are subject to the requirements of this part.

(b) *Microorganisms automatically included on the Inventory.* Microorganisms that are not intergeneric are automatically included on the Inventory.

(c) *Microorganisms not subject to this part.* The following microorganisms are not subject to this part, either because they are not subject to jurisdiction under the Act or are not subject to reporting under section 5 of the Act.

(1) Any microorganism which would be excluded from the definition of "chemical substance" in section 3 of the Act and § 720.3(e) of this chapter.

(2) Any microbial mixture as defined in § 725.3. This exclusion applies only to a microbial mixture as a whole and not to any microorganisms and other chemical substances which are part of the microbial mixture.

(3) Any microorganism that is manufactured and processed solely for export if the following conditions are met:

(i) The microorganism is labeled in accordance with section 12(a)(1)(B) of the Act, when the microorganism is distributed in commerce.

(ii) The manufacturer and processor can document at the commencement of manufacturing or processing that the person to whom the microorganism will be distributed intends to export it or process it solely for export as defined in § 721.3 of this chapter.

§ 725.12 Identification of microorganisms for Inventory and other listing purposes.

To identify and list microorganisms on the Inventory, both taxonomic designations and supplemental information will be used. The supplemental information required in paragraph (b) of this section will be used to specifically describe an individual microorganism on the Inventory. Submitters must provide the supplemental information required by paragraph (b) of this section to the extent necessary to enable a microorganism to be accurately and unambiguously identified on the Inventory.

(a) *Taxonomic designation.* The taxonomic designation of a microorganism must be provided for the donor organism and the recipient microorganism to the level of strain, as appropriate. These designations must be substantiated by a letter from a culture collection, literature references, or the results of tests conducted for the purpose of taxonomic classification. Upon EPA's request to the submitter, data supporting the taxonomic designation must be provided to EPA. The genetic history of the recipient microorganism should be documented back to the isolate from which it was derived.

(b) *Supplemental information.* The supplemental information described in paragraphs (b)(1) and (b)(2) of this section is required to the extent that it enables a microorganism to be accurately and unambiguously identified.

(1) *Phenotypic information.* Phenotypic information means pertinent traits that result from the interaction of a microorganism's genotype and the environment in which it is intended to be used and may include intentionally added biochemical and physiological traits.

(2) *Genotypic information.* Genotypic information means the pertinent and distinguishing genotypic characteristics of a microorganism, such as the

identity of the introduced genetic material and the methods used to construct the reported microorganism. This also may include information on the vector construct, the cellular location, and the number of copies of the introduced genetic material.

§ 725.15 Determining applicability when microorganism identity or use is confidential or uncertain.

(a) *Consulting EPA.* Persons intending to conduct activities involving microorganisms may determine their obligations under this part by consulting the Inventory or the microorganisms and uses specified in § 725.239 or in subpart M of this part. This section establishes procedures for EPA to assist persons in determining whether the microorganism or the use is listed on the Inventory, in § 725.239 or in subpart M of this part.

(1) Confidential identity or use. In some cases it may not be possible to directly determine if a specific microorganism is listed, because portions of that entry may contain generic information to protect confidential business information (CBI). If any portion of the *microorganism's identity or use* has been claimed as CBI, that portion does not appear on the public version of the Inventory, in § 725.239 or in subpart M of this part. Instead, it is contained in a confidential version held in EPA's Confidential Business Information Center (CBIC). The public versions contain generic information which masks the confidential business information. A person who intends to conduct an activity involving a microorganism or use whose entry is described with generic information will need to inquire of EPA whether the unreported microorganism or use is on the confidential version.

(2) Uncertain microorganism identity. The current state of scientific knowledge leads to some imprecision in describing a microorganism. As the state of knowledge increases, EPA will be developing policies to determine whether one microorganism is equivalent to another. Persons intending to conduct activities involving microorganisms may inquire of EPA whether the microorganisms they intend to manufacture, import, or process are equivalent to specific microorganisms described on the Inventory, in § 725.239, or in subpart M of this part.

(b) *Requirement of bona fide intent.* (1) EPA will answer the inquiries described in paragraph (a) of this section only if the Agency determines that the person has a *bona fide* intent to conduct the activity for which reporting is required or for which any exemption may apply.

(2) To establish a *bona fide* intent to manufacture, import, or process a microorganism, the person who intends to manufacture, import, or process the microorganism must submit the following information in writing to the Office of Pollution Prevention and Toxics, Document Control Officer, 7407, 1200 Pennsylvania Ave., NW., Washington, DC 20460, ATTN: BIOTECH *bona fide* submission.

(i) Taxonomic designations and supplemental information required by § 725.12.

(ii) A signed statement certifying that the submitter intends to manufacture, import, or process the microorganism for commercial purposes.

(iii) A description of research and development activities conducted with the microorganism to date, demonstration of the submitter's ability to produce or obtain the microorganism from a foreign manufacturer, and the purpose for which the person will manufacture, import, or process the microorganism.

(iv) An indication of whether a related microorganism was previously reviewed by EPA to the extent known by the submitter.

(v) A specific description of the major intended application or use of the microorganism.

(c) If an importer or processor cannot provide all the information required by paragraph (b) of this section, because it is claimed as confidential business information by its foreign manufacturer or supplier, the foreign manufacturer or supplier may supply the information directly to EPA.

(d) EPA will review the information submitted by the manufacturer, importer, or processor under this paragraph to determine whether that person has shown a *bona fide* intent to manufacture, import, or process the

microorganism. If necessary, EPA will compare this information to the information requested for the confidential microorganism under § 725.85(b)(3)(iii).

(e) In order for EPA to make a conclusive determination of the microorganism's status, the proposed manufacturer, importer, or processor must show a *bona fide* intent to manufacture, import, or process the microorganism and must provide sufficient information to establish identity unambiguously. After sufficient information has been provided, EPA will inform the manufacturer, importer, or processor whether the microorganism is subject to this part and if so, which sections of this part apply.

(f) If the microorganism is found on the confidential version of the Inventory, in § 725.239 or in subpart M of this part, EPA will notify the person(s) who originally reported the microorganism that another person (whose identity will remain confidential, if so requested) has demonstrated a *bona fide* intent to manufacture, import, or process the microorganism and therefore was told that the microorganism is on the Inventory, in § 725.239, or in subpart M of this part.

(g) A disclosure to a person with a *bona fide* intent to manufacture, import, or process a particular microorganism that the microorganism is on the Inventory, in § 725.239, or in subpart M of this part will not be considered a public disclosure of confidential business information under section 14 of the Act.

(h) EPA will answer an inquiry on whether a particular microorganism is subject to this part within 30 days after receipt of a complete submission under paragraph (b) of this section.

§ 725.17 Consultation with EPA.

Persons may consult with EPA, either in writing or by telephone, about their obligations under this part. Written consultation is preferred. Written inquiries should be sent to the following address: Environmental Assistance Division (7408), Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460, ATTN: Biotechnology Notice Consultation. Persons wishing to consult with EPA by telephone should call (202) 554-1404; hearing impaired TDD (202) 554-0551 or e-mail: *TSCA-Hotline@epamail.epa.gov*.

Subpart B—Administrative Procedures

§ 725.20 Scope and purpose.

This subpart describes general administrative procedures applicable to all persons who submit MCANs and exemption requests to EPA under section 5 of the Act for microorganisms.

§ 725.25 General administrative requirements.

(a) *General.* (1) Each person who is subject to the notification provisions of this part must complete, sign, and submit a MCAN or exemption request containing the information as required for the appropriate submission under this part. Except as otherwise provided, each submission must include all referenced attachments. All information in the submission (unless certain attachments appear in the open scientific literature) must be in English. All information submitted must be true and correct.

(2) In addition to specific information required, the submitter should submit all information known to or reasonably ascertainable by the submitter that would permit EPA to make a reasoned evaluation of the human health and environmental effects of the microorganism and any microbial mixture or article that may contain the microorganism.

(b) *Certification.* Persons submitting MCANs and exemption requests to EPA under this part, and material related to their reporting obligations under this part, must attach the following statement to any information submitted to EPA. This statement must be signed and dated by an authorized official of the submitter:

I certify that to the best of my knowledge and belief: The company named in this submission intends to manufacture, import, or process for a commercial purpose, other than in small quantities solely for research and development, the microorganism identified in this submission. All information provided in this submission is complete and truthful as of the date of submission. I am including

with this submission all test data in my possession or control and a description of all other data known to or reasonably ascertainable by me as required by 40 CFR 725.160 or 725.260.

(c) *Where to submit information under this part.* Persons submitting MCANs and exemption requests to EPA under this part, and material related to their reporting obligations under this part, must send them to: TSCA Document Processing Center (7407), Rm. L–100, Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

(d) *General requirements for submission of data.* (1) Submissions under this part must include the information described in § 725.155, 725.255, 725.355, or 725.455, as appropriate, to the extent such information is known to or reasonably ascertainable by the submitter.

(2) In accordance with § 725.160 or 725.260, as appropriate, the submission must also include any test data in the submitter's possession or control and descriptions of other data which are known to or reasonably ascertainable by the submitter and which concern the health and environmental effects of the microorganism.

(e) *Agency or joint submissions.* (1) A manufacturer or importer may designate an agent to submit the MCAN or exemption request. Both the manufacturer or importer and the agent must sign the certification required in paragraph (b) of this section.

(2) A manufacturer or importer may authorize another person (e.g., a foreign manufacturer or supplier, or a toll manufacturer) to report some of the information required in the MCAN or exemption request to EPA on its behalf. If separate portions of a joint submission are not submitted together, the submitter must indicate which information will be supplied by another person and identify that person. The manufacturer or importer and any other person supplying the information must sign the certification required by paragraph (b) of this section.

(3) If EPA receives a submission which does not include the information required, which the submitter indicates that it has authorized another person to provide, the review period will not begin until EPA receives all of the required information.

(f) *Microorganisms subject to a section 4 test rule.* (1) Except as provided in paragraph (f)(3) of this section, if a person intends to manufacture or import a new microorganism which is subject to the notification requirements of this part, and the microorganism is subject to a test rule promulgated under section 4 of the Act before the notice is submitted, section 5(b)(1) of the Act requires the person to submit the test data required by the testing rule with the notice. The person must submit the data in the form and manner specified in the test rule and in accordance with § 725.160. If the person does not submit the test data, the submission is incomplete and EPA will follow the procedures in § 725.33.

(2) If EPA has granted the submitter an exemption under section 4(c) of the Act from the requirement to conduct tests and submit data, the person may not file a MCAN or TERA until EPA receives the test data.

(3) If EPA has granted the submitter an exemption under section 4(c) of the Act and if another person previously has submitted the test data to EPA, the exempted person may either submit the test data or provide the following information as part of the notice:

(i) The name, title, and address of the person who submitted the test data to EPA.

(ii) The date the test data were submitted to EPA.

(iii) A citation for the test rule.

(iv) A description of the exemption and a reference identifying it.

(g) *Microorganisms subject to a section 5(b)(4) rule.* (1) If a person:

(i) Intends to manufacture or import a microorganism which is subject to the notification requirements of this part and which is subject to a rule issued under section 5(b)(4) of the Act; and

(ii) Is not required by a rule issued under section 4 of the Act to submit test data for the microorganism before the filing of a submission, the person must submit to EPA data described in paragraph (g)(2) of this section at the time the submission is filed.

(2) Data submitted under paragraph (g)(1) of this section must be data which the person submitting the notice believes show that the manufacture, processing, distribution in commerce, use, and disposal of the microorganism, or any combination of such activities, will not present an unreasonable risk of injury to health or the environment.

(h) *Data that need not be submitted.* Specific data requirements are listed in subparts D, E, F, G, and L of this part. The following is a list of data that need not be submitted under this part:

(1) Data previously submitted to EPA. (i) A person need not submit any data previously submitted to EPA with no claims of confidentiality if the new submission includes: the office or person to whom the data were submitted; the date of submission; and, if appropriate, a standard literature citation as specified in § 725.160(a)(3)(ii).

(ii) For data previously submitted to EPA with a claim of confidentiality, the person must resubmit the data with the new submission and any claim of confidentiality, under § 725.80.

(2) Efficacy data. This part does not require submission of any data related solely to product efficacy. However, including efficacy data will improve EPA's ability to assess the benefits of the use of the microorganism. This does not exempt a person from submitting any of the data specified in § 725.160 or 725.260.

(3) Non-U.S. exposure data. This part does not require submission of any data which relates only to exposure of humans or the environment outside the United States. This does not exclude nonexposure data such as data on health effects (including epidemiological studies), ecological effects, physical and chemical properties, or environmental fate characteristics.

§ 725.27 Submissions.

Each person who is required to submit information under this part must submit the information in the form and manner set forth in the appropriate subpart.

(a) Requirements specific to MCANs are described in §§ 725.150 through 725.160.

(b) Requirements specific to TERAs are described in §§ 725.250 through 725.260.

(c) Requirements specific to test marketing exemptions (TMEs) are described in §§ 725.350 and 725.355.

(d) Requirements specific to Tier I and Tier II exemptions for certain general commercial uses are described in §§ 725.424 through 725.470.

(e) Additional requirements specific to significant new uses for microorganisms are described at § 725.950.

§ 725.28 Notice that submission is not required.

When EPA receives a MCAN or exemption request, EPA will review it to determine whether the microorganism is subject to the requirements of this part. If EPA determines that the microorganism is not subject to these requirements, EPA will notify the submitter that section 5 of the Act does not prevent the manufacture, import, or processing of the microorganism and that the submission is not needed.

§ 725.29 EPA acknowledgement of receipt of submission.

(a) EPA will acknowledge receipt of each submission by sending the submitter a letter that identifies the number assigned to each MCAN or exemption request and the date on which the review period begins. The review period will begin on the date the MCAN or exemption request is received by the Office of Pollution Prevention and Toxics Document Control Officer.

(b) The acknowledgement does not constitute a finding by EPA that the submission is in compliance with this part.

§ 725.32 Errors in the submission.

(a) Within 30 days of receipt of the submission, EPA may request that the submitter remedy errors in the submission. The following are examples of such errors:

(1) Failure to date the submission.

(2) Typographical errors that cause data to be misleading or answers to any questions to be unclear.

(3) Contradictory information.

(4) Ambiguous statements or information.

(b) In the request to correct the submission, EPA will explain the action which the submitter must take to correct the submission.

(c) If the submitter fails to correct the submission within 15 days of receipt of the request, EPA may extend the review period.

§ 725.33 Incomplete submissions.

(a) A submission under this part is not complete, and the review period does not begin, if:

(1) The wrong person files the submission.

(2) The submitter does not attach and sign the certification statement as required by § 725.25(b).

(3) Some or all of the information in the submission or any attachments are not in English, except for published scientific literature.

(4) The submitter does not provide information that is required by sections 5(d)(1)(B) and (C) of the Act and § 725.160 or 725.260, as appropriate.

(5) The submitter does not provide information required by § 725.25, 725.155, 725.255, 725.355, or 725.455, as appropriate, or indicate that it is not known to or reasonably ascertainable by the submitter.

(6) The submitter has asserted confidentiality claims and has failed to:

(i) Submit a second copy of the submission with all confidential information deleted for the public file, as required by § 725.80(b)(2).

(ii) Comply with the substantiation requirements as described in § 725.94.

(7) The submitter does not include any information required by section 5(b)(1) of the Act and pursuant to a rule promulgated under section 4 of the Act, as required by § 725.25(f).

(8) The submitter does not submit data which the submitter believes show that the microorganism will not present an unreasonable risk of injury to health or the environment, if EPA has listed the microorganism under section 5(b)(4) of the Act, as required in § 725.25(g).

(9) For MCANs, the submitter does not remit the fees required by § 700.45(b)(1) or (b)(2)(vi) of this chapter.

(b)(1) If EPA receives an incomplete submission under this part, the Director, or a designee, will notify the submitter within 30 days of receipt that the submission is incomplete and that the review period will not begin until EPA receives a complete submission.

(2) If EPA obtains additional information during the review period for any submission that indicates the original submission was incomplete, the Director, or a designee, may declare the submission incomplete within 30 days after EPA obtains the additional information and so notify the submitter.

(c) The notification that a submission is incomplete under paragraph (b) of this section will include:

(1) A statement of the basis of EPA's determination that the submission is incomplete.

(2) The requirements for correcting the incomplete submission.

(3) Information on procedures under paragraph (d) of this section for filing objections to the determination or requesting modification of the requirements for completing the submission.

(d) Within 10 days after receipt of notification by EPA that a submission is incomplete, the submitter may file written objections requesting that EPA accept the submission as complete or modify the requirements necessary to complete the submission.

(e)(1) EPA will consider the objections filed by the submitter. The Director, or a designee, will determine whether the submission was complete or incomplete, or whether to modify the requirements for completing the submission. EPA will notify the submitter in writing of EPA's response within 10 days of receiving the objections.

(2) If the Director, or a designee, determines, in response to the objection, that the submission was complete, the review period will be deemed suspended on the date EPA declared the submission incomplete, and will resume on the date that the submission is declared complete. The submitter need not correct the submission as EPA originally requested. If EPA can complete its review within the review period beginning on the date of the submission, the Director, or a designee, may inform the submitter that the

running of the review period will resume on the date EPA originally declared it incomplete.

(3) If the Director, or a designee, modifies the requirements for completing the submission or concurs with EPA's original determination, the review period will begin when EPA receives a complete submission.

(f) If EPA discovers at any time that a person submitted materially false or misleading statements in information submitted under this part, EPA may find that the submission was incomplete from the date it was submitted, and take any other appropriate action.

§ 725.36 New information.

(a) During the review period, if a submitter possesses, controls, or knows of new information that materially adds to, changes, or otherwise makes significantly more complete the information included in the MCAN or exemption request, the submitter must send that information to the address listed in § 725.25(c) within 10 days of receiving the new information, but no later than 5 days before the end of the review period.

(b) The new submission must clearly identify the submitter, the MCAN or exemption request to which the new information is related, and the number assigned to that submission by EPA, if known to the submitter.

(c) If the new information becomes available during the last 5 days of the review period, the submitter must immediately inform the EPA contact for that submission by telephone of the new information.

§ 725.40 Notice in the Federal Register.

(a) *Filing of* FEDERAL REGISTER *notice.* After EPA receives a MCAN or an exemption request under this part, EPA will issue a notice in the FEDERAL REGISTER including the information specified in paragraph (b) of this section.

(b) *Contents of notice.* (1) In the public interest, the specific microorganism identity listed in the submission will be published in the FEDERAL REGISTER unless the submitter has claimed the microorganism identity confidential. If the submitter claims confidentiality, a generic name will be published in accordance with § 725.85.

(2) The categories of use of the microorganism will be published as reported in the submission unless this information is claimed confidential. If confidentiality is claimed, the generic information which is submitted under § 725.88 will be published.

(3) A list of information submitted in accordance with § 725.160(a), 725.255, 725.260, 725.355, or 725.455, as appropriate, will be published.

(4) The submitter's identity will be published, unless the submitter has claimed it confidential.

(c) *Publication of exemption decisions.* Following the expiration of the appropriate review period for the exemption request, EPA will issue a notice in the FEDERAL REGISTER indicating whether the request has been approved or denied and the reasons for the decision.

§ 725.50 EPA review.

(a) *MCANs.* The review period specified in section 5(a) of the Act for MCANs runs for 90 days from the date the Document Control Officer receives a complete submission, or the date EPA determines the submission is complete under § 725.33, unless the Agency extends the review period under section 5(c) of the Act and § 725.56.

(b) *Exemption requests.* The review period starts on the date the Document Control Officer receives a complete exemption request, or the date EPA determines the request is complete under § 725.33, unless the Agency extends the review period under § 725.56. The review periods for exemption requests run as follows:

(1) *TERAs.* The review period for TERAs is 60 days.

(2) *TMEs.* The review period for TMEs is 45 days.

(3) *Tier II exemption requests.* The review period for Tier II exemption requests is 45 days.

§ 725.54 Suspension of the review period.

(a) A submitter may voluntarily suspend the running of the review period if the Director, or a designee, agrees. If the Director does not agree, the review period will continue to run, and EPA will notify the submitter. A submitter

may request a suspension at any time during the review period. The suspension must be for a specified period of time.

(b) A request for suspension may be made in writing to the address listed in § 725.25(c). The suspension also may be made orally, including by telephone, to the submitter's EPA contact for that submission. EPA will send the submitter a written confirmation that the suspension has been granted.

(1) An oral request may be granted for no longer than 15 days. To obtain a longer suspension, the Document Control Officer for the Office of Pollution Prevention and Toxics must receive written confirmation of the oral request. The review period is suspended as of the date of the oral request.

(2) If the submitter has not made a previous oral request, the running of the review period is suspended as of the date of receipt of the written request by the Document Control Officer for the Office of Pollution Prevention and Toxics.

§ 725.56 Extension of the review period.

(a) At any time during the review period, EPA may unilaterally determine that good cause exists to extend the review period specified for MCANs, or the exemption requests.

(b) If EPA makes such a determination, EPA:

(1) Will notify the submitter that EPA is extending the review period for a specified length of time and state the reasons for the extension.

(2) For MCANs, EPA may issue a notice for publication in the FEDERAL REGISTER which states that EPA is extending the review period and gives the reasons for the extension.

(c) The total period of the extension may be for a period of up to the same length of time as specified for each type of submission in § 725.50. If the initial extension is for less than the total time allowed, EPA may make additional extensions. However, the sum of the extensions may not exceed the total allowed.

(d) The following are examples of situations in which EPA may find that good cause exists for extending the review period:

(1) EPA has reviewed the submission and is seeking additional information.

(2) EPA has received significant additional information during the review period.

(3) The submitter has failed to correct a submission after receiving EPA's request under § 725.32.

(4) EPA has reviewed the submission and determined that there is a significant possibility that the microorganism will be regulated under section 5(e) or section 5(f) of the Act, but EPA is unable to initiate regulatory action within the initial review period.

§ 725.60 Withdrawal of submission by the submitter.

(a) A submitter may withdraw a submission during the review period. A statement of withdrawal must be made in writing to the address listed in § 725.25(c). The withdrawal is effective upon receipt of the statement by the Document Control Officer.

(b) If a manufacturer, importer, or processor who withdrew a submission later resubmits a submission for the same microorganism, a new review period begins.

§ 725.65 Recordkeeping.

(a) *General provisions.* (1) Any person who submits a notice under this part must retain documentation of information in the submission, including:

(i) Any data in the submitter's possession or control; and

(ii) Records of production volume for the first 3 years of manufacture, import, or processing.

(2) Any person who submits a notice under this part must retain documentation of the date of commencement of testing, manufacture, import, or processing.

(3) Any person who is exempt from some or all of the reporting requirements of this part must retain documentation that supports the exemption.

(4) All information required by this section must be retained for 3 years from the date of commencement of each activity for which records are required under this part.

(b) *Specific requirements.* In addition to the requirements of paragraph (a) of

this section, specific recordkeeping requirements included in certain subparts must also be followed.

(1) Additional recordkeeping requirements for activities conducted inside a structure are set forth in §725.235(h).

(2) Additional recordkeeping requirements for TERAs are set forth in §725.250(f).

(3) Additional recordkeeping requirements for TMEs are set forth in §725.350(c).

(4) Additional recordkeeping requirements for Tier I exemptions under subpart G of this part are set forth in §725.424(a)(5).

(5) Additional recordkeeping requirements for Tier II exemptions under subpart G of this part are set forth in §725.450(d).

(6) Additional recordkeeping requirements for significant new uses of microorganisms reported under subpart L of this part are set forth in §725.850. Recordkeeping requirements may also be included when a microorganism and significant new use are added to subpart M of this part.

§725.67 Applications to exempt new microorganisms from this part.

(a) *Submission.* (1) Any manufacturer or importer of a new microorganism may request, under section 5(h)(4) of the Act, an exemption, in whole or in part, from this part by sending a Letter of Application to the Chief, New Chemicals Branch, Chemical Control Division, Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

(2) *General provisions.* The Letter of Application should provide information to show that any activities affected by the requested exemption will not present an unreasonable risk of injury to health or the environment. This information should include data described in the following paragraphs.

(i) The effects of the new microorganism on health and the environment.

(ii) The magnitude of exposure of human beings and the environment to the new microorganism.

(iii) The benefits of the new microorganism for various uses and the availability of substitutes for such uses.

(iv) The reasonably ascertainable economic consequences of granting or denying the exemption, including effects on the national economy, small business, and technological innovation.

(3) *Specific requirements.* In addition to the requirements of paragraph (a)(2) of this section, the specific information requirements of the relevant subpart under which the exemption is sought should be met.

(i) *Exemption from MCAN reporting under subpart D.* Information requirements are set forth in §§725.155 and 725.160.

(ii) *Exemption from TERA reporting under subpart E.* Information requirements are set forth in §§725.255 and 725.260.

(iii) *Listing a recipient microorganism as eligible for exemption under subpart G.* Information regarding the following criteria should be addressed in an application to list a recipient microorganism under §725.420:

(A) Identification and classification of the microorganism using available genotypic and phenotypic information;

(B) Information to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on health or the environment;

(C) A history of safe commercial use for the microorganism;

(D) Commercial uses indicating that the microorganism products might be subject to TSCA;

(E) Studies which indicate the potential for the microorganism to cause adverse effects to health or the environment; and

(F) Studies which indicate the survival characteristics of the microorganism in the environment.

(b) *Processing of the Letter of Application by EPA*—(1) *Grant of the Application.* If, after consideration of the Letter of Application and any other relevant information available to EPA, the Assistant Administrator for Prevention, Pesticides and Toxic Substances makes a preliminary determination that the new microorganism will not present an unreasonable risk of injury to health or the environment, the Assistant Administrator will propose a rule to grant the exemption

using the applicable procedures in part 750 of this chapter.

(2) *Denial of the application.* If the Assistant Administrator decides that the preliminary determination described in paragraph (b)(1) of this section cannot be made, the application will be denied by sending the applicant a written statement with the Assistant Administrator's reasons for denial.

(c) *Processing of the exemption*—(1) *Unreasonable risk standard.* Granting a section 5(h)(4) exemption requires a determination that the activities will not present an unreasonable risk of injury to health or the environment.

(i) An unreasonable risk determination under the Act is an administrative judgment that requires balancing of the harm to health or the environment that a chemical substance may cause and the magnitude and severity of that harm, against the social and economic effects on society of EPA action to reduce that harm.

(ii) A determination of unreasonable risk under section 5(h)(4) of the Act will examine the reasonably ascertainable economic and social consequences of granting or denying the exemption after consideration of the effect on the national economy, small business, technological innovation, the environment, and public health.

(2) *Grant of the exemption.* The exemption will be granted if the Assistant Administrator determines, after consideration of all relevant evidence presented in the rulemaking proceeding described in paragraph (b)(1) of this section, that the new microorganism will not present an unreasonable risk of injury to health or the environment.

(3) *Denial of the exemption.* The exemption will be denied if the Assistant Administrator determines, after consideration of all relevant evidence presented in the rulemaking proceeding described in paragraph (b)(1) of this section, that the determination described in paragraph (c)(2) of this section cannot be made. A final decision terminating the rulemaking proceeding will be published in the FEDERAL REGISTER.

§ 725.70 Compliance.

(a) Failure to comply with any provision of this part is a violation of section 15 of the Act (15 U.S.C. 2614).

(b) A person who manufactures or imports a microorganism before a MCAN is submitted and the MCAN review period expires is in violation of section 15 of the Act even if that person was not required to submit the MCAN under § 725.105.

(c) Using a microorganism which a person knew or had reason to know was manufactured, processed, or distributed in commerce in violation of section 5 of the Act or this part is a violation of section 15 of the Act (15 U.S.C. 2614).

(d) Failure or refusal to establish and maintain records or to permit access to or copying of records, as required by the Act, is a violation of section 15 of the Act (15 U.S.C. 2614).

(e) Failure or refusal to permit entry or inspection as required by section 11 of the Act is a violation of section 15 of the Act (15 U.S.C. 2614).

(f) Violators may be subject to the civil and criminal penalties in section 16 of the Act (15 U.S.C. 2615) for each violation. Persons who submit materially misleading or false information in connection with the requirements of any provision of this part may be subject to penalties calculated as if they never filed their submissions.

(g) EPA may seek to enjoin the manufacture or processing of a microorganism in violation of this part or act to seize any microorganism manufactured or processed in violation of this part or take other actions under the authority of section 7 of the Act (15 U.S.C. 2606) or section 17 of the Act (15 U.S.C. 2616).

§ 725.75 Inspections.

EPA will conduct inspections under section 11 of the Act to assure compliance with section 5 of the Act and this part, to verify that information required by EPA under this part is true and correct, and to audit data submitted to EPA under this part.

Subpart C—Confidentiality and Public Access to Information

§ 725.80 General provisions for confidentiality claims.

(a) A person may assert a claim of confidentiality for any information submitted to EPA under this part. However,

(1) Any person who asserts a claim of confidentiality for portions of the specific microorganism identity must provide the information as described in § 725.85.

(2) Any person who asserts a claim of confidentiality for a use of a microorganism must provide the information as described in § 725.88.

(3) Any person who asserts a claim of confidentiality for information contained in a health and safety study of a microorganism must provide the information described in § 725.92.

(b) Any claim of confidentiality must accompany the information when it is submitted to EPA.

(1) When a person submits any information under this part, including any attachments, for which claims of confidentiality are made, the claim(s) must be asserted by circling the specific information which is claimed and marking the page on which that information appears with an appropriate designation such as "trade secret," "TSCA CBI," or "confidential business information."

(2) If any information is claimed confidential, the person must submit two copies of the document including the claimed information.

(i) One copy of the document must be complete. In that copy, the submitter must mark the information which is claimed as confidential in the manner prescribed in paragraph (b)(1) of this section.

(ii) The second copy must be complete except that all information claimed as confidential in the first copy must be deleted. EPA will place the second copy in the public file.

(iii) If the submitter does not provide the second copy, the submission is incomplete and the review period does not begin to run until EPA receives the second copy, in accordance with § 725.33.

(iv) Any information contained within the copy submitted under paragraph (b)(2)(ii) of this section which has been in the public file for more than 30 days will be presumed to be in the public domain, notwithstanding any assertion of confidentiality made under this section.

(3) A person who submits information to EPA under this part must reassert a claim of confidentiality and substantiate the claim each time the information is submitted to EPA.

(c) Any person asserting a claim of confidentiality under this part must substantiate each claim in accordance with the requirements in § 725.94.

(d) EPA will disclose information that is subject to a claim of confidentiality asserted under this section only to the extent permitted by the Act, this subpart, and part 2 of this title.

(e) If a submitter does not assert a claim of confidentiality for information at the time it is submitted to EPA, EPA may make the information public and place it in the public file without further notice to the submitter.

§ 725.85 Microorganism identity.

(a) *Claims applicable to the period prior to commencement of manufacture or import for general commercial use*—(1) *When to make a claim.* (i) A person who submits information to EPA under this part may assert a claim of confidentiality for portions of the specific microorganism identity at the time of submission of the information. This claim will apply only to the period prior to the commencement of manufacture or import for general commercial use.

(ii) A person who submits information to EPA under this part must reassert a claim of confidentiality and substantiate the claim each time the information is submitted to EPA. For example, if a person claims certain information confidential in a TERA submission and wishes the same information to remain confidential in a subsequent TERA or MCAN submission, the person must reassert and resubstantiate the claim in the subsequent submission.

(2) *Assertion of claim.* (i) A submitter may assert a claim of confidentiality

only if the submitter believes that public disclosure prior to commencement of manufacture or import for general commercial use of the fact that anyone is initiating research and development activities pertaining to the specific microorganism or intends to manufacture or import the specific microorganism for general commercial use would reveal confidential business information. Claims must be substantiated in accordance with the requirements of § 725.94(a).

(ii) If the submission includes a health and safety study concerning the microorganism and if the claim for confidentiality with respect to the specific identity is denied in accordance with § 725.92(c), EPA will deny a claim asserted under paragraph (a) of this section.

(3) *Development of generic name.* Any person who asserts a claim of confidentiality for portions of the specific microorganism identity under this paragraph must provide one of the following items at the time the submission is filed:

(i) The generic name which was accepted by EPA in the prenotice consultation conducted under paragraph (a)(4) of this section.

(ii) One generic name that is only as generic as necessary to protect the confidential identity of the particular microorganism. The name should reveal the specific identity to the maximum extent possible. The generic name will be subject to EPA review and approval.

(4) *Determination by EPA.* (i) Any person who intends to assert a claim of confidentiality for the specific identity of a new microorganism may seek a determination by EPA of an appropriate generic name for the microorganism before filing a submission. For this purpose, the person should submit to EPA:

(A) The specific identity of the microorganism.

(B) A proposed generic name(s) which is only as generic as necessary to protect the confidential identity of the new microorganism. The name(s) should reveal the specific identity of the microorganism to the maximum extent possible.

(ii) Within 30 days, EPA will inform the submitter either that one of the proposed generic names is adequate or that none is adequate and further consultation is necessary.

(5) *Use of generic name.* If a submitter claims microorganism identity as confidential under paragraph (a) of this section, and if the submitter complies with paragraph (a)(2) of this section, EPA will issue for publication in the FEDERAL REGISTER notice described in § 725.40 the generic name proposed by the submitter or one agreed upon by EPA and the submitter.

(b) *Claims applicable to the period after commencement of manufacture or import for general commercial use*—(1) *Maintaining claim.* Any claim of confidentiality under paragraph (a) of this section is applicable only until the microorganism is manufactured or imported for general commercial use and becomes eligible for inclusion on the Inventory. To maintain the confidential status of the microorganism identity when the microorganism is added to the Inventory, a submitter must reassert the confidentiality claim and substantiate the claim in the notice of commencement of manufacture required under § 725.190.

(i) A submitter may not claim the microorganism identity confidential for the period after commencement of manufacture or import for general commercial use unless the submitter claimed the microorganism identity confidential under paragraph (a) of this section in the MCAN submitted for the microorganism.

(ii) A submitter may claim the microorganism identity confidential for the period after commencement of manufacture or import for general commercial use if the submitter did not claim the microorganism identity confidential under paragraph (a) of this section in any TERA submitted for the microorganism, but subsequently did claim microorganism identity confidential in the MCAN submitted for the microorganism.

(2) *Assertion of claim.* (i) A person who believes that public disclosure of the fact that anyone manufactures or imports the microorganism for general commercial use would reveal confidential business information may assert a

claim of confidentiality under paragraph (b) of this section.

(ii) If the notice includes a health and safety study concerning the new microorganism, and if the claim for confidentiality with respect to the microorganism identity is denied in accordance with §725.92(c), EPA will deny a claim asserted under paragraph (b) of this section.

(3) *Requirements for assertion.* Any person who asserts a confidentiality claim for microorganism identity must:

(i) Comply with the requirements of paragraph (a)(3) of this section regarding submission of a generic name.

(ii) Agree that EPA may disclose to a person with a *bona fide* intent to manufacture or import the microorganism the fact that the particular microorganism is included on the confidential Inventory for purposes of notification under section 5(a)(1)(A) of the Act.

(iii) Have available and agree to furnish to EPA upon request the taxonomic designations and supplemental information required by §725.12.

(iv) Provide a detailed written substantiation of the claim, in accordance with the requirements of §725.94(b).

(4) *Denial of claim.* If the submitter does not meet the requirements of paragraph (b) of this section, EPA will deny the claim of confidentiality.

(5) *Acceptance of claim.* (i) EPA will publish a generic name on the public Inventory if:

(A) The submitter asserts a claim of confidentiality in accordance with this paragraph.

(B) No claim for confidentiality of the microorganism identity as part of a health and safety study has been denied in accordance with part 2 of this title or §725.92.

(ii) Publication of a generic name on the public Inventory does not create a category for purposes of the Inventory. Any person who has a *bona fide* intent to manufacture or import a microorganism which is described by a generic name on the public Inventory may submit an inquiry to EPA under §725.15(b) to determine whether the particular microorganism is included on the confidential Inventory.

(iii) Upon receipt of a request described in §725.15(b), EPA may require the submitter who originally asserted confidentiality for a microorganism to submit to EPA the information listed in paragraph (b)(3)(iii) of this section.

(iv) Failure to submit any of the information required under paragraph (b)(3)(iii) of this section within 10 calendar days of receipt of a request by EPA under paragraph (b) of this section will constitute a waiver of the original submitter's confidentiality claim. In this event, EPA may place the specific microorganism identity on the public Inventory without further notice to the original submitter.

(6) *Use of generic name on the public Inventory.* If a submitter asserts a claim of confidentiality under paragraph (b) of this section, EPA will examine the generic microorganism name proposed by the submitter.

(i) If EPA determines that the generic name proposed by the submitter is only as generic as necessary to protect the confidential identity of the particular microorganism, EPA will place that generic name on the public Inventory.

(ii) If EPA determines that the generic name proposed by the submitter is more generic than necessary to protect the confidential identity, EPA will propose in writing, for review by the submitter, an alternative generic name that will reveal the identity of the microorganism to the maximum extent possible.

(iii) If the generic name proposed by EPA is acceptable to the submitter, EPA will place that generic name on the public Inventory.

(iv) If the generic name proposed by EPA is not acceptable to the submitter, the submitter must explain in detail why disclosure of that generic name would reveal confidential business information and propose another generic name which is only as generic as necessary to protect the confidential identity of the microorganism. If EPA does not receive a response from the submitter within 30 days after the submitter receives the proposed name, EPA will place EPA's chosen generic name on the public Inventory. If the submitter does provide the information requested, EPA will review the response. If the submitter's proposed generic name is acceptable, EPA will

publish that generic name on the public Inventory. If the submitter's proposed generic name is not acceptable, EPA will notify the submitter of EPA's choice of a generic name. Thirty days after this notification, EPA will place the chosen generic name on the public Inventory.

§ 725.88 Uses of a microorganism.

(a) *Assertion of claim.* A person who submits information to EPA under this part on the categories or proposed categories of use of a microorganism may assert a claim of confidentiality for this information.

(b) *Requirements for claim.* A submitter that asserts such a claim must:

(1) Report the categories or proposed categories of use of the microorganism.

(2) Provide, in nonconfidential form, a description of the uses that is only as generic as necessary to protect the confidential business information. The generic use description will be included in the FEDERAL REGISTER notice described in § 725.40.

(c) *Generic use description.* The person must submit the information required by paragraph (b) of this section by describing the uses as precisely as possible, without revealing the information which is claimed confidential, to disclose as much as possible how the use may result in human exposure to the microorganism or its release to the environment.

§ 725.92 Data from health and safety studies of microorganisms.

(a) *Information other than specific microorganism identity.* Except as provided in paragraph (b) of this section, EPA will deny any claim of confidentiality with respect to information included in a health and safety study of a microorganism, unless the information would disclose confidential business information concerning:

(1) Processes used in the manufacture or processing of a microorganism.

(2) Information which is not in any way related to the effects of a microorganism on health or the environment, such as, the name of the submitting company, cost or other financial data, product development or marketing plans, and advertising plans, for which the person submits a claim of confidentiality in accordance with § 725.80.

(b) *Microorganism identity*—(1) *Claims applicable to the period prior to commencement of manufacture or import for general commercial use.* A claim of confidentiality for the period prior to commencement of manufacture or import for general commercial use for the specific identity of a microorganism for which a health and safety study was submitted must be asserted in conjunction with a claim asserted under § 725.85(a). The submitter must substantiate each claim in accordance with the requirements of § 725.94(a).

(2) *Claims applicable to the period after commencement of manufacture or import for general commercial use.* To maintain the confidential status of the specific identity of a microorganism for which a health and safety study was submitted after commencement of manufacture or import for general commercial use, the claim must be reasserted and substantiated in conjunction with a claim under § 725.85(b). The submitter must substantiate each claim in accordance with the requirements of § 725.94(b).

(c) *Denial of confidentiality claim.* EPA will deny a claim of confidentiality for microorganism identity under paragraph (b) of this section, unless:

(1) The information would disclose processes used in the manufacture or processing of a microorganism.

(2) The microorganism identity is not necessary to interpret a health and safety study.

(d) *Use of generic names.* When EPA discloses a health and safety study containing a microorganism identity, which the submitter has claimed confidential, and if the Agency has not denied the claim under paragraph (c) of this section, EPA will identify the microorganism by the generic name selected under § 725.85.

§ 725.94 Substantiation requirements.

(a) *Claims applicable to the period prior to commencement of manufacture or import for general commercial use*—(1) *MCAN, TME, Tier I certification, and Tier II exemption request requirements.* Any person who submits a MCAN, TME, Tier I certification, or Tier II exemption request should strictly limit

confidentiality claims to that information which is confidential and proprietary to the business.

(i) If any information in the submission is claimed as confidential business information, the submitter must substantiate each claim by submitting written answers to the questions in paragraphs (c), (d), and (e) of this section at the time the person submits the information.

(ii) If the submitter does not provide written substantiation as required in paragraph (a)(1)(i) of this section, the submission will be considered incomplete and the review period will not begin in accordance with §725.33.

(2) *TERA requirements.* Any person who submits a TERA, should strictly limit confidentiality claims to that information which is confidential and proprietary to the business. If any information in such a submission is claimed as confidential business information, the submitter must have available for each of those claims, and agree to furnish to EPA upon request, written answers to the questions in paragraphs (d) and (e) of this section.

(b) *Claims applicable to the period after commencement of manufacture or import for general commercial use.* (1) If a submitter claimed portions of the microorganism identity confidential in the MCAN and wants the identity to be listed on the confidential Inventory, the claim must be reasserted and substantiated at the time the Notice of Commencement (NOC) is submitted under §725.190. Otherwise, EPA will list the specific microorganism identity on the public Inventory.

(2) The submitter must substantiate the claim for confidentiality of the microorganism identity by answering all of the questions in paragraphs (c), (d), and (e) in this section. In addition, the following questions must be answered:

(i) What harmful effects to the company's or institution's competitive position, if any, would result if EPA publishes on the Inventory the identity of the microorganism? How could a competitor use such information given the fact that the identity of the microorganism otherwise would appear on the TSCA Inventory with no link between the microorganism and the company or institution? How substantial would the harmful effects of disclosure be? What is the causal relationship between the disclosure and the harmful effects?

(ii) Has the identity of the microorganism been kept confidential to the extent that competitors do not know it is being manufactured or imported for general commercial use by anyone?

(c) *General questions.* The following questions must be answered in detail for each confidentiality claim:

(1) For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

(2) Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

(3) Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

(4) Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential.

(i) Advertising or promotional materials for the microorganism or the resulting end product?

(ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?

(iii) Professional or trade publications?

(iv) Any other media available to the public or to competitors?

(v) Patents?

(vi) Local, State, or Federal agency public files?

(5) Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

(6) For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

(7) If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

(d) *Microorganism identity and production method.* If confidentiality claims are asserted for the identity of the microorganism or information on how the microorganism is produced, the following questions must be answered:

(1) Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

(2) Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

(3) For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

(e) *Health and safety studies of microorganisms.* If confidentiality claims are asserted for information in a health or safety study of a microorganism, the following questions must be answered:

(1) Would the disclosure of the information claimed confidential reveal: confidential process information, or information unrelated to the effects of the microorganism on health and the environment. Describe the causal connection between the disclosure and harm.

(2) Does the company or institution assert that disclosure of the microorganism identity is not necessary to interpret any health and safety studies which have been submitted? If so, explain how a less specific identity would be sufficient to interpret the studies.

§ 725.95 Public file.

All information submitted, including any health and safety study of a microorganism and other supporting documentation, will become part of the public file for that submission, unless such materials are claimed confidential. In addition, EPA may add materials to the public file, unless such materials are claimed confidential. Any of the nonconfidential material described in this subpart will be available for public inspection in the TSCA Public Docket Office, Rm. NE-B607, 401 M St., SW., Washington, DC, between the hours of noon to 4 p.m., Monday through Friday, excluding legal holidays.

Subpart D—Microbial Commercial Activities Notification Requirements

§ 725.100 Scope and purpose.

(a) This subpart establishes procedures for submission of a notice to EPA under section 5(a) of the Act for persons who manufacture, import, or process microorganisms for commercial purposes. This notice is called a Microbial Commercial Activity Notice (MCAN). It is expected that MCANs will in general only be submitted for microorganisms intended for general commercial use. Persons who manufacture, import, or process a microorganism in small quantities solely for research and development as defined in § 725.3 are not required to submit a notice to EPA. Persons who manufacture, import, or process a microorganism for research and development activities that do not fit the definition of small quantities solely for research and development may nonetheless qualify for more limited reporting requirements in Subpart E, including the TERA which can be used for review of research and development involving environmental release.

(b) Persons subject to MCAN submission are described in § 725.105.

(c) Exclusions and exemptions specific to MCAN submissions are described in § 725.110.

(d) Submission requirements applicable specifically to MCANs are described at § 725.150.

(e) Data requirements for MCANs are set forth in §§ 725.155 and 725.160.

(f) EPA review procedures specific to MCANs are set forth in § 725.170.

(g) Subparts A through C of this part apply to any MCAN submitted under this subpart.

§ 725.105 Persons who must report.

(a) *Manufacturers of new microorganisms.* (1) MCAN submission is required for any person who intends to manufacture for commercial purposes in the United States a new microorganism. Exclusions are described in § 725.110.

(2) If a person contracts with a manufacturer to produce or process a new microorganism and the manufacturer produces or processes the microorganism exclusively for that person, and that person specifies the identity of the microorganism, and controls the total amount produced and the basic technology for the plant process, then that person must submit the MCAN. If it is unclear who must report, EPA should be contacted to determine who must submit the MCAN.

(3) Only manufacturers that are incorporated, licensed, or doing business in the United States may submit a MCAN.

(b) *Importers of new microorganisms.* (1) MCAN submission is required for a person who intends to import into the United States for commercial purposes a new microorganism. Exclusions are described in § 725.110.

(2) When several persons are involved in an import transaction, the MCAN must be submitted by the principal importer. If no one person fits the principal importer definition in a particular transaction, the importer should contact EPA to determine who must submit the MCAN for that transaction.

(3) Except as otherwise provided in paragraph (b)(4) of this section, the provisions of this subpart D apply to each person who submits a MCAN for a new microorganism which such person intends to import for a commercial purpose. In addition, each importer must comply with paragraph (b)(4) of this section.

(4) EPA will hold the principal importer, or the importer that EPA determines must submit the MCAN when there is no principal importer under paragraph (b)(2) of this section, liable for complying with this part, for completing the MCAN, and for the completeness and truthfulness of all information which it submits.

(c) *Manufacturers, importers, or processors of microorganisms for a significant new use.* MCAN submission is required for any person who intends to manufacture, import, or process for commercial purposes a microorganism identified as having one or more significant new uses in subpart M of this part, and who intends either to engage in a designated significant new use of the microorganism or intends to distribute it in commerce. Persons excluded from reporting on significant new uses of microorganisms and additional procedures for reporting are described in subpart L of this part.

§ 725.110 Persons not subject to this subpart.

Persons are not subject to the requirements of this subpart for the following activities:

(a) Manufacturing, importing, or processing solely for research and development microorganisms that meet the requirements for an exemption under subpart E of this part.

(b) Manufacturing, importing, or processing microorganisms for test marketing activities which have been granted an exemption under subpart F of this part.

(c) Manufacturing or importing new microorganisms under the conditions of a Tier I or Tier II exemption under subpart G of this part.

§ 725.150 Procedural requirements for this subpart.

General requirements for all MCANs under this part are contained in subparts A through C of this part. In addition, the following requirements apply to MCANs submitted under this subpart:

(a) *When to submit a MCAN.* A MCAN must be submitted at least 90 calendar days prior to manufacturing or importing a new microorganism and at least

90 calendar days prior to manufacturing, importing, or processing a microorganism for a significant new use.

(b) *Section 5(b) of the Act.* The submitter must comply with any applicable requirement of section 5(b) of the Act for the submission of test data.

(c) *Contents of a MCAN.* Each person who submits a MCAN under this subpart must provide the information and test data described in §§ 725.155 and 725.160.

(d) *Recordkeeping.* Each person who submits a MCAN under this subpart must comply with the recordkeeping requirements of § 725.65.

§ 725.155 Information to be included in the MCAN.

(a) Each person who is required by this part to submit a MCAN must include the information specified in paragraphs (c) through (h) of this section, to the extent it is known to or reasonably ascertainable by that person. However, no person is required to include information which relates solely to exposure of humans or ecological populations outside of the United States.

(b) Each person should also submit, in writing, all other information known to or reasonably ascertainable by that person that would permit EPA to make a reasoned evaluation of the health and environmental effects of the microorganism, or any microbial mixture or article, including information on its effects on humans, animals, plants, and other microorganisms, and in the environment. The information to be submitted under this subpart includes the information listed in paragraphs (c) through (h) of this section relating to the manufacture, processing, distribution in commerce, use, and disposal of the new microorganism.

(c) *Submitter identification.* (1) The name and headquarters address of the submitter.

(2) The name, address, and office telephone number (including area code) of the principal technical contact representing the submitter.

(d) *Microorganism identity information.* Persons must submit sufficient information to allow the microorganism to be accurately and unambiguously identified for listing purposes as required by § 725.12.

(1) *Description of the recipient microorganism and the new microorganism.* (i) Data substantiating the taxonomy of the recipient microorganism and the new microorganism to the level of strain, as appropriate. In lieu of data, EPA will accept a letter from a culture collection substantiating taxonomy, provided EPA, upon request to the submitter, may have access to the data supporting the taxonomic designation.

(ii) Information on the morphological and physiological features of the new microorganism.

(iii) Other specific data by which the new microorganism may be uniquely identified for Inventory purposes.

(2) *Genetic construction of the new microorganism.* (i) Data substantiating the taxonomy of the donor organism(s). In lieu of data, EPA will accept a letter from a culture collection substantiating taxonomy, provided EPA, upon request to the submitter, may have access to the data supporting the taxonomic designation.

(ii) Description of the traits for which the new microorganism has been selected or developed and other traits known to have been added or modified.

(iii) A detailed description of the genetic construction of the new microorganism, including the technique used to modify the microorganism (e.g., fusion of cells, injection of DNA, electroporation or chemical poration, or methods used for induced mutation and selection). The description should include, for example, a description of the introduced genetic material, including any regulatory sequences and structural genes and the products of those genes; how the introduced genetic material is expected to affect behavior of the recipient; expression, alteration, and stability of the introduced genetic material; methods for vector construction and introduction; and a description of the regulatory and structural genes that are components of the introduced genetic material, including genetic maps of the introduced sequences.

(3) *Phenotypic and ecological characteristics.* (i) Habitat, geographical distribution, and source of the recipient microorganism.

(ii) Survival and dissemination under relevant environmental conditions including a description of methods for detecting the new or recipient microorganism(s) in the environment and the sensitivity limit of detection for these techniques.

(iii) A description of anticipated biological interactions with and effects on target organisms and other organisms such as competitors, prey, hosts, symbionts, parasites, and pathogens; a description of host range; a description of pathogenicity, infectivity, toxicity, virulence, or action as a vector of pathogens; and capacity for genetic transfer under laboratory and relevant environmental conditions.

(iv) A description of anticipated involvement in biogeochemical or biological cycling processes, involvement in rate limiting steps in mineral or nutrient cycling, or involvement in inorganic compounds cycling (such as possible sequestration or transformation of heavy metals).

(e) *Byproducts.* A description of the byproducts resulting from the manufacture, processing, use, and disposal of the new microorganism.

(f) *Total production volume.* The estimated maximum amount of the new microorganism intended to be manufactured or imported during the first year of production and the estimated maximum amount to be manufactured or imported during any consecutive 12-month period during the first 3 years of production. This estimate may be by weight or volume and should include an estimation of viability (i.e., viable cells per unit volume or colony forming units per unit dry weight).

(g) *Use information.* A description of intended categories of use by function and application, the estimated percent of production volume devoted to each category of use, and the percent of the new microorganism in the formulation for each commercial or consumer use.

(h) *Worker exposure and environmental release.* (1) For sites controlled by the submitter:

(i) The identity of sites where the new microorganism will be manufactured, processed, or used. For purposes of this section, the site for a person who imports a new microorganism is the site of the operating unit within the person's organization which is directly responsible for importing the new microorganism and which controls the import transaction. The import site may in some cases be the organization's headquarters office in the United States.

(ii) A process description of each manufacture, processing, and use operation, which includes a diagram of the major unit operations and conversions, the identity and entry point of all feedstocks, and the identity of any possible points of release of the new microorganism from the process, including a description of all controls, including engineering controls, used to prevent such releases.

(iii) Worker exposure information, including worker activities, physical form of process streams which contain the new microorganism to which workers may be exposed, the number of workers, and the duration of activities.

(iv) Information on release of the new microorganism to the environment, including the quantity and media of release and type of control technology used.

(v) A narrative description of the intended transport of the new microorganism, including the means of transport, containment methods to be used during transport, and emergency containment procedures to be followed in case of accidental release.

(vi) Procedures for disposal of any articles, waste, clothing, or other equipment involved in the activity, including procedures for inactivation of the new microorganism, containment, disinfection, and disposal of contaminated items.

(2) For sites not controlled by the submitter, a description of each type of processing and use operation involving the new microorganism, including identification of the estimated number of processing or use sites, situations in which worker exposure to and/or environmental release of the new microorganism will occur, the number of workers exposed and the duration of exposure; procedures for transport of the new microorganism and for disposal, including procedures for inactivation of the new microorganism; and control measures which limit worker exposure and environmental release.

§ 725.160 Submission of health and environmental effects data.

(a) *Test data on the new microorganism in the possession or control of the submitter.* (1) Except as provided in § 725.25(h), and in addition to the information required by § 725.155(d)(3), each MCAN must contain all test data in the submitter's possession or control which are related to the effects on health or the environment of any manufacture, processing, distribution in commerce, use, or disposal of the new microorganism or any microbial mixture or article containing the new microorganism, or any combination of such activities. This includes test data concerning the new microorganism in a pure culture or formulated form as used or as intended to be used in one of the activities listed above.

(2) A full report or standard literature citation must be submitted for the following types of test data:

(i) Health effects data.

(ii) Ecological effects data.

(iii) Physical and chemical properties data.

(iv) Environmental fate characteristics.

(v) Monitoring data and other test data related to human exposure to or environmental release of the new microorganism.

(3)(i) If the data do not appear in the open scientific literature, the submitter must provide a full report. A full report includes the experimental methods and materials, results, discussion and data analysis, conclusions, references, and the name and address of the laboratory that developed the data.

(ii) If the data appear in the open scientific literature, the submitter need only provide a standard literature citation. A standard literature citation includes author, title, periodical name, date of publication, volume, and page numbers.

(4)(i) If a study, report, or test is incomplete when a person submits a MCAN, the submitter must identify the nature and purpose of the study; name and address of the laboratory developing the data; progress to date; types of data collected, significant preliminary results; and anticipated completion date.

(ii) If a test or experiment is completed before the MCAN review period ends, the person must submit the study, report, or test, as specified in paragraph (a)(3)(i) of this section, to the address listed in § 725.25(c) within 10 days of receiving it, but no later than 5 days before the end of the review period. If the test or experiment is completed during the last 5 days of the review period, the submitter must immediately inform its EPA contact for that submission by telephone.

(5) For test data in the submitter's possession or control which are not listed in paragraph (a)(2) of this section, a person is not required to submit a complete report. The person must submit a summary of the data. If EPA so requests, the person must submit a full report within 10 days of the request, but no later than 5 days before the end of the review period.

(6) All test data described under paragraph (a) of this section are subject to these requirements, regardless of their age, quality, or results.

(b) *Other data concerning the health and environmental effects of the new microorganism that are known to or reasonably ascertainable by the submitter.* (1) Except as provided in § 725.25(h), and in addition to the information required by § 725.155(c)(3), any person who submits a MCAN must describe the following data, including any data from a health and safety study of a microorganism, if the data are related to effects on health or the environment of any manufacture, processing, distribution in commerce, use, or disposal of the microorganism, of any microbial mixture or article containing the new microorganism, or of any combination of such activities:

(i) Any data, other than test data, in the submitter's possession or control.

(ii) Any data, including test data, which are not in the submitter's possession or control, but which are known to or reasonably ascertainable by the submitter. For the purposes of this section, data are known to or reasonably ascertainable by the submitter if the data are known to any of its employees or other agents who are associated with the research and development, test marketing, or commercial marketing of the microorganism.

(2) Data that must be described include data concerning the new microorganism in a pure culture or formulated form as used or as intended to be used in one of the activities listed in paragraph (b)(1) of this section.

(3) The description of data reported under paragraph (b) of this section must include:

(i) If the data appear in the open scientific literature, a standard literature citation, which includes the author, title, periodical name, date of publication, volume, and pages.

(ii) If the data are not available in the open scientific literature, a description of the type of data and summary of the results, if available, and the names and addresses of persons the submitter believes may have possession or control of the data.

(4) All data described in paragraph (b) of this section are subject to these requirements, regardless of their age, quality, or results; and regardless of whether they are complete at the time the MCAN is submitted.

§ 725.170 EPA review of the MCAN.

General procedures for review of all submissions under this part are contained in §§ 725.28 through 725.60. In addition, the following procedures apply to EPA review of MCANs submitted under this subpart:

(a) Length of the review period. The MCAN review period specified in section 5(a) of the Act runs for 90 days from the date the Document Control Officer for the Office of Pollution Prevention and Toxics receives a complete MCAN, or the date EPA determines the MCAN is complete under § 725.33, unless the Agency extends the period under section 5(c) of the Act and § 725.56.

(b) Notice of expiration of MCAN review period. (1) EPA will notify the submitter that the MCAN review period has expired or that EPA has completed its review of the MCAN. Expiration of the review period does not constitute EPA approval or certification of the new microorganism, and does not mean that EPA may not take regulatory action against the microorganism in the future.

(2) After expiration of the MCAN review period, in the absence of regulatory action by EPA under section 5(e), 5(f), or 6(a) of the Act, the submitter may manufacture or import the microorganism even if the submitter has not received notice of expiration.

(3) Early notification that EPA has completed its review does not permit commencement of manufacture or import prior to the expiration of the 90-day MCAN review period.

(c) No person submitting a MCAN in response to the requirements of this subpart may manufacture, import, or process a microorganism subject to this subpart until the review period, including all extensions and suspensions, has expired.

§ 725.190 Notice of commencement of manufacture or import.

(a) *Applicability.* Any person who commences the manufacture or import of a new microorganism for nonexempt, commercial purposes for which that person previously submitted a section 5(a) notice under this part must submit a notice of commencement (NOC) of manufacture or import.

(b) *When to report.* (1) If manufacture or import for nonexempt, commercial purposes begins on or after May 27, 1997, the submitter must submit the NOC to EPA no later than 30 calendar days after the first day of such manufacture or import.

(2) If manufacture or import for nonexempt, commercial purposes began or will begin before May 27, 1997, the submitter must submit the NOC by May 27, 1997.

(3) Submission of an NOC prior to the commencement of manufacture or import is a violation of section 15 of the Act.

(c) *Information to be reported.* The NOC must contain the following information: Specific microorganism identity, MCAN number, and the date when manufacture or import commences. If the person claimed microorganism identity confidential in the MCAN, and wants the identity to be listed on the confidential Inventory, the claim must be reasserted and resubstantiated in accordance with § 725.85(b). Otherwise, EPA will list the specific microorganism identity on the public Inventory.

(d) *Where to submit.* NOCs should be submitted to the address listed in § 725.25(c).

Subpart E—Exemptions for Research and Development Activities

§ 725.200 Scope and purpose.

(a) This subpart describes exemptions from the reporting requirements under subpart D of this part for research and development activities involving microorganisms.

(b) In lieu of complying with subpart D of this part, persons described in § 725.205 may submit a TSCA Experimental Release Application (TERA) for research and development activities involving microorganisms or otherwise comply with this subpart.

(c) Exemptions from part 725 are provided at §§ 725.232, 725.234, and 725.238.

(d) Submission requirements specific for TERAs are described at § 725.250.

(e) Data requirements for TERAs are set forth in §§ 725.255 and 725.260.

(f) EPA review procedures specific for TERAs are set forth in §§ 725.270 and 725.288.

(g) Subparts A through C of this part apply to any submission under this subpart.

§ 725.205 Persons who may report under this subpart.

(a) Commercial research and development activities involving new microorganisms or significant new uses of microorganisms are subject to reporting under this part unless they qualify for an exemption under this part.

(b) Commercial purposes for research and development means that the activities are conducted with the purpose of obtaining an immediate or eventual commercial advantage for the researcher and would include:

(1) All research and development activities which are funded directly, in whole or in part, by a commercial entity regardless of who is actually conducting the research. Indications that the research and development activities are funded directly, in whole or in part, may include, but are not limited to:

(i) Situations in which a commercial entity contracts directly with a university or researcher; or

(ii) Situations in which a commercial entity gives a conditional grant where the commercial entity holds patent rights, or establishes a joint venture where the commercial entity holds patent or licensing rights; or

(iii) Any other situation in which the commercial entity intends to obtain an immediate or eventual commercial advantage for the commercial entity and/or the researcher.

(2) Research and development activities that are not funded directly by a commercial entity, if the researcher intends to obtain an immediate or eventual commercial advantage. Indications that the researcher intends to obtain an immediate or eventual commercial advantage may include, but are not limited to:

(i) The research is directed toward developing a commercially viable improvement of a product already on the market; or

(ii) The researcher has sought or is seeking commercial funding for the purpose of developing a commercial application; or

(iii) The researcher or university has sought or is seeking a patent to protect a commercial application which the research is developing; or

(iv) Other evidence that the researcher is aware of a commercial application for the research and has directed the research toward developing that application.

(c) Certain research and development activities involving microorganisms subject to jurisdiction under the Act are exempt from reporting under this part. A person conducting research and development activities which meet the conditions for the exemptions described in §§ 725.232, 725.234, or 725.238 is exempt from TERA reporting under this subpart.

(d) A microorganism is not exempt from reporting under subpart D of this part if any amount of the microorganism, including as part of a mixture, is processed, distributed in commerce, or used, for any commercial purpose other than research and development.

(e) Quantities of the inactivated microorganism, or mixtures or articles containing the inactivated microorganism, remaining after completion of research and development activities may be disposed of as a waste in accordance with applicable Federal, State, and local regulations.

(f) A person who manufactures, imports, or processes a microorganism solely for research and development is not required to comply with the requirements of this section if:

(1) The person is manufacturing a microbial pesticide identified in §172.45(c), or

(2) The person is manufacturing a microbial pesticide for which an Experimental Use Permit is required, pursuant to §172.3; or

(3) The person is manufacturing a microbial pesticide for which a notification or an Experimental Use Permit is not required to be submitted.

§725.232 Activities subject to the jurisdiction of other Federal programs or agencies.

This part does not apply to any research and development activity that meets all of the following conditions.

(a) The microorganism is manufactured, imported, or processed solely for research and development activities.

(b) There is no intentional testing of a microorganism outside of a structure, as structure is defined in §725.3.

(c)(1) The person receives research funds from another Federal agency, and the funds are awarded on the condition that the research will be conducted in accordance with the relevant portions of the NIH Guidelines, or

(2) A Federal agency or program otherwise imposes the legally binding requirement that the research is to be conducted in accordance with relevant portions of the NIH Guidelines.

§725.234 Activities conducted inside a structure.

A person who manufactures, imports, or processes a microorganism is not subject to the reporting requirements under subpart D of this part if all of the following conditions are met:

(a) The microorganism is manufactured, imported, or processed solely for research and development activities.

(b) The microorganism is used by, or directly under the supervision of, a technically qualified individual, as defined in §725.3. The technically qualified individual must maintain documentation of the procedures selected to comply with paragraph (d) of this section and must ensure that the procedures are used.

(c) There is no intentional testing of a microorganism outside of a structure, as structure is defined in §725.3.

(d) Containment and/or inactivation controls. (1) Selection and use of containment and/or inactivation controls inside a structure for a particular microorganism shall take into account the following:

(i) Factors relevant to the organism's ability to survive in the environment.

(ii) Potential routes of release in air, solids and liquids; in or on waste materials and equipment; in or on people, including maintenance and custodial personnel; and in or on other organisms, such as insects and rodents.

(iii) Procedures for transfer of materials between facilities.

(2) The technically qualified individual's selection of containment and/or inactivation controls shall be approved and certified by an authorized official (other than the TQI) of the institution that is conducting the test prior to the commencement of the test.

(3) Records shall be developed and maintained describing the selection and use of containment and/or inactivation controls, as specified in §725.235(c). These records, which must be maintained at the location where the research and development activity is being conducted, shall be submitted to EPA upon written request and within the time frame specified in EPA's request.

(4) Subsequent to EPA review of records in accordance with paragraph (d)(3) of this section, changes to the containment/inactivation controls selected under paragraph (d)(1) of this section must be made upon EPA order. Failure to comply with EPA's order shall result in automatic loss of eligibility for an exemption under this section.

(e) The manufacturer, importer, or processor notifies all persons in its employ or to whom it directly distributes the microorganism, who are engaged in experimentation, research, or analysis on the microorganism, including the manufacture, processing, use, transport, storage, and disposal of the microorganism associated with research and development activities, of

any risk to health, identified under § 725.235(a), which may be associated with the microorganism. The notification must be made in accordance with § 725.235(b).

§ 725.235 Conditions of exemption for activities conducted inside a structure.

(a) *Determination of risks.* To determine whether notification under § 725.234(e) is required, the manufacturer, importer, or processor must do one of the following:

(1) For research conducted in accordance with the NIH Guidelines, the manufacturer, importer, or processor must meet the conditions laid out at IV-B-4-d of the NIH Guidelines; or

(2) For all other research conducted in accordance with § 725.234, the manufacturer, importer, or processor must review and evaluate the following information to determine whether there is reason to believe there is any risk to health which may be associated with the microorganism:

(i) Information in its possession or control concerning any significant adverse reaction of persons exposed to the microorganism which may reasonably be associated with such exposure.

(ii) Information provided to the manufacturer, importer, or processor by a supplier or any other person concerning a health risk believed to be associated with the microorganism.

(iii) Health and environmental effects data in its possession or control concerning the microorganism.

(iv) Information on health effects which accompanies any EPA rule or order issued under TSCA section 4, 5, or 6 of the Act that applies to the microorganism and of which the manufacturer, importer, or processor has knowledge.

(b) *Notification to employees and others.* (1) The manufacturer, importer, or processor must notify the persons identified in § 725.234(e) by means of a container labeling system, conspicuous placement of notices in areas where exposure may occur, written notification to each person potentially exposed, or any other method of notification which adequately informs persons of health risks which the manufacturer, importer, or processor has reason to believe may be associated with the microorganism, as determined under paragraph (a) of this section.

(2) If the manufacturer, importer, or processor distributes a microorganism manufactured, imported, or processed under this section to persons not in its employ, the manufacturer, importer, or processor must in written form:

(i) Notify those persons that the microorganism is to be used only for research and development purposes and the requirements of § 725.234 are to be met.

(ii) Provide the notice of health risks specified in paragraph (b)(1) of this section.

(3) The adequacy of any notification under this section is the responsibility of the manufacturer, importer, or processor.

(c) *Recordkeeping.* (1) For research conducted in accordance with the NIH Guidelines, a person who manufactures, imports, or processes a microorganism under this section must retain the following records:

(i) Documentation that the NIH Guidelines have been adhered to. Such documentation shall include:

(A) For experiments subject to Institutional Biosafety Committee review, or notification simultaneous with initiation of the experiment, the information submitted for review or notification, along with standard laboratory records, shall satisfy the recordkeeping requirements specified in § 725.234(d)(3).

(B) For experiments exempt from Institutional Biosafety Committee review or notification simultaneous with initiation of the experiment, documentation of the exemption, along with standard laboratory records, shall satisfy the recordkeeping requirement specified in § 725.234(d)(3).

(ii) Documentation of how the following requirements are satisfied under the NIH Guidelines:

(A) Copies or citations to information reviewed and evaluated to determine the need to make any notification of risk.

(B) Documentation of the nature and method of notification of risk, including copies of any labels or written notices used.

(C) The names and addresses of any persons other than the manufacturer,

importer, or processor to whom the substance is distributed, the identity of the microorganism, the amount distributed, and copies of the notifications required.

(2) For all other research conducted in accordance with § 725.234, a person who manufacturers, imports, or processes a microorganism under this section, must maintain the following records:

(i) Records describing selection and use of containment and/or inactivation controls required by § 725.234(d)(3) and certification by an authorized official required by § 725.234(d)(2) for each microorganism.

(ii) Copies or citations to information reviewed and evaluated under paragraph (a) of this section to determine the need to make any notification of risk.

(iii) Documentation of the nature and method of notification under paragraph (b)(1) of this section, including copies of any labels or written notices used.

(iv) The names and addresses of any persons other than the manufacturer, importer, or processor to whom the substance is distributed, the identity of the microorganism, the amount distributed, and copies of the notifications required under paragraph (b)(2) of this section.

§ 725.238 Activities conducted outside a structure.

(a) *Exemption.* (1) Research and development activities involving intentional testing in the environment of certain microorganisms listed in § 725.239 may be conducted without prior review by EPA if all of the conditions of this section and § 725.239 are met.

(2) The research and development activity involving a microorganism listed in § 725.239 must be conducted by, or directly under the supervision of, a technically qualified individual, as defined in § 725.3.

(b) *Certification.* To be eligible for the exemption under this section, a manufacturer or importer must submit to EPA prior to initiation of the activity a document signed by an authorized official containing the following information:

(1) Name, address, and telephone number of the manufacturer or importer.

(2) Location, estimated duration, and planned start date of the test.

(3) Certification of the following:

(i) Compliance with the conditions of the exemption specified for the microorganism in § 725.239.

(ii) If state and/or local authorities have been notified of the activity, evidence of notification.

(c) *Recordkeeping.* Persons who conduct research and development activities under this section must comply with the recordkeeping requirements of § 725.65 and retain documentation that supports their compliance with the requirements of this section and the specific requirements for the microorganism listed in § 725.239.

§ 725.239 Use of specific microorganisms in activities conducted outside a structure.

(a) *Bradyrhizobium japonicum.* To qualify for an exemption under this section, all of the following conditions must be met for a test involving *Bradyrhizobium japonicum:*

(1) *Characteristics of recipient microorganism.* The recipient microorganism is limited to strains of *Bradyrhizobium japonicum.*

(2) *Modification of traits.* (i) The introduced genetic material must meet the criteria for poorly mobilizable listed in § 725.421(c).

(ii) The introduced genetic material must consist only of the following components:

(A) The structural gene(s) of interest, which have the following limitations:

(*1*) For structural genes encoding marker sequences, the gene is limited to the *aadH* gene, which confers resistance to the antibiotics streptomycin and spectinomycin.

(*2*) For traits other than antibiotic resistance, the structural gene must be limited to the genera *Bradyrhizobium* and *Rhizobium.*

(B) The regulatory sequences permitting the expression of solely the gene(s) of interest.

(C) Associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites.

(D) The vector nucleotide sequences needed for vector transfer.

(E) The vector nucleotide sequences needed for vector maintenance.

(3) *Limitations on exposure.* (i) The test site area must be no more than 10 terrestrial acres.

(ii) The technically qualified individual must select appropriate methods to limit the dissemination of modified *Bradyrhizobium japonicum.*

(b) *Rhizobium meliloti.* To qualify for an exemption under this section, all of the following conditions must be met for a test involving *Rhizobium meliloti:*

(1) *Characteristics of recipient microorganism.* The recipient microorganism is limited to strains of *Rhizobium meliloti.*

(2) *Modification of traits.* (i) The introduced genetic material must meet the criteria for poorly mobilizable listed in § 725.421(c) of this part.

(ii) The introduced genetic material must consist only of the following components:

(A) The structural gene(s) of interest, which have the following limitations:

(*1*) For structural genes encoding marker sequences, the gene is limited to the *aadH* gene, which confers resistance to the antibiotics streptomycin and spectinomycin.

(*2*) For traits other than antibiotic resistance, the structural gene must be limited to the genera *Bradyrhizobium* and *Rhizobium.*

(B) The regulatory sequences permitting the expression of solely the gene(s) of interest.

(C) Associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites.

(D) The vector nucleotide sequences needed for vector transfer.

(E) The vector nucleotide sequences needed for vector maintenance.

(3) *Limitations on exposure.* (i) The test site area must be no more than 10 terrestrial acres.

(ii) The technically qualified individual must select appropriate methods to limit the dissemination of modified *Rhizobium meliloti.*

§ 725.250 Procedural requirements for the TERA.

General requirements for all submissions under this part are contained in subparts A through C of this part. In addition, the following requirements apply to TERAs submitted under this subpart:

(a) When to submit the TERA. Each person who is eligible to submit a TERA under this subpart must submit the TERA at least 60 calendar days before the person intends to initiate the proposed research and development activity.

(b) Contents of the TERA. Each person who submits a TERA under this subpart must provide the information and test data described in §§ 725.255 and 725.260. In addition, the submitter must supply sufficient information to enable EPA to evaluate the effects of all activities for which approval is requested.

(c) A person may submit a TERA for one or more microorganisms and one or more research and development activities, including a research program.

(d) EPA will either approve the TERA, with or without conditions, or disapprove it under procedures established in this subpart.

(e) The manufacturer, importer, or processor who receives a TERA approval must comply with all terms of the approval, as well as conditions described in the TERA, and remains liable for compliance with all terms and conditions, regardless of who conducts the research and development activity. Any person conducting the research and development activity approved under the TERA must comply with all terms of the TERA approval, as well as the conditions described in the TERA.

(f) Recordkeeping. Persons submitting a TERA must comply with the recordkeeping requirements of § 725.65. In addition, the following requirements apply to TERAs:

(1) Each person submitting a TERA under this part must retain documentation of information contained in the TERA for a period of 3 years from the date that the results of the study are submitted to the Agency.

(2) Summaries of all data, conclusions, and reports resulting from the conduct of the research and development activity under the TERA must be submitted to the EPA address identified in §725.25(c) within 1 year of the termination of the activity.

§ 725.255 Information to be included in the TERA.

(a) To review a TERA, EPA must have sufficient information to permit a reasoned evaluation of the health and environmental effects of the planned test in the environment. The person seeking EPA approval must submit all information known to or reasonably ascertainable by the submitter on the microorganism(s) and the research and development activity, including information not listed in paragraphs (c), (d), and (e) of this section that the person believes will be useful for EPA's risk assessment. The TERA must be in writing and must include at least the information described in the following paragraphs.

(b) When specific information is not submitted, an explanation of why such information is not available or not applicable must be included.

(c) Persons applying for a TERA, must include the submitter identification and microorganism identity information required for MCANs in §725.155(c), (d)(1), and (d)(2).

(d) Persons applying for a TERA must submit phenotypic and ecological characteristics information required in §725.155(d)(3) as it relates directly to the conditions of the proposed research and development activity.

(e) Persons applying for a TERA must also submit the following information about the proposed research and development activity:

(1) *A detailed description of the proposed research and development activity.* (i) The objectives and significance of the activity and a rationale for testing the microorganisms in the environment.

(ii) Number of microorganisms released (including viability per volume if applicable) and the method(s) of application or release.

(iii) Characteristics of the test site(s), including location, geographical, physical, chemical, and biological features, proximity to human habitation or activity, and description of site characteristics that would influence dispersal or confinement.

(iv) Target organisms (if the microorganism(s) to be tested has an intended target), including identification of each target organism and anticipated mechanism and result of interaction.

(v) Planned start date and duration of each activity.

(vi) If State and/or local authorities have been notified of the activity, evidence of notification.

(2) *Information on monitoring, confinement, mitigation, and emergency termination procedures.* (i) Confinement procedures for the activity, access and security measures, and procedures for routine termination of the activity.

(ii) Mitigation and emergency procedures.

(iii) Measures to detect and control potential adverse effects.

(iv) Name of principal investigator and chief of site personnel responsible for emergency procedures.

(v) Personal protective equipment, engineering controls, and procedures to be followed to minimize dispersion of the microorganism(s) by people, machinery, or equipment.

(vi) Procedures for disposal of any articles, waste, clothing, machinery, or other equipment involved in the experimental release, including methods for inactivation of the microorganism(s), containment, disinfection, and disposal of contaminated items.

§ 725.260 Submission of health and environmental effects data.

Each TERA must contain all available data concerning actual or potential effects on health or the environment of the new microorganism that are in the possession or control of the submitter and a description of other data known to or reasonably ascertainable by the submitter that will permit a reasoned evaluation of the planned test in the environment. The data must be reported in the manner described in §725.160(a)(3) and (b)(3).

§ 725.270 EPA review of the TERA.

General procedures for review of all submissions under this part are contained in §§ 725.28 through 725.60. In addition, the following procedures apply to EPA review of applications submitted under this subpart:

(a) *Length of the review period.* (1) The review period for the TERA will be 60 days from the date the Document Control Officer for the Office of Pollution Prevention and Toxics receives a complete TERA, or the date EPA determines the TERA is complete under § 725.33, unless EPA finds good cause for an extension under § 725.56.

(2) A submitter shall not proceed with the research and development activity described in the TERA unless and until EPA provides written approval of the TERA. A submitter may receive early approval if a review is completed in less than 60 days.

(b) *EPA decision regarding proposed TERA activity.* (1) A decision concerning a TERA under this subpart will be made by the Administrator, or a designee.

(2) If EPA determines that the proposed research and development activity for the microorganism does not present an unreasonable risk of injury to health or the environment, EPA will notify the submitter that the TERA is approved and that the submitter can proceed with the proposed research and development activity described in the TERA.

(3) EPA may include requirements and conditions in its approval of the TERA that would be stated in the TERA approval under paragraph (c) of this section.

(4) If EPA concludes that it cannot determine that the proposed research and development activity described in the TERA will not present an unreasonable risk of injury to health or the environment, EPA will deny the TERA and will provide reasons for the denial in writing.

(c) *TERA approval.* (1) A TERA approval issued by EPA under this section is legally binding on the TERA submitter.

(2) When EPA approves a TERA, the submitter must conduct the research and development activity only as described in the TERA and in accordance with any requirements and conditions prescribed by EPA in its approval of the TERA.

(3) Any person who fails to conduct the research and development activity as described in the TERA and in accordance with any requirements and conditions prescribed by EPA in its approval of the TERA under this section, shall be in violation of sections 5 and 15 of the Act and be subject to civil and criminal penalties under section 16 of the Act.

§ 725.288 Revocation or modification of TERA approval.

(a) *Significant questions about risk.* (1) If, after approval of a TERA under this subpart, EPA receives information which raises significant questions about EPA's determination that the activity does not present an unreasonable risk of injury to health or the environment, EPA will notify the submitter in writing of those questions.

(2) The submitter may, within 10 days of receipt of EPA's notice, provide in writing additional information or arguments concerning the significance of the questions and whether EPA should modify or revoke the approval of the TERA.

(3) After considering any such information and arguments, EPA will decide whether to change its determination regarding approval of the TERA.

(i) If EPA determines that the activity will not present an unreasonable risk of injury to health or the environment, it will notify the submitter in writing. To make this finding, EPA may prescribe additional conditions which must be followed by the submitter.

(ii) If EPA determines that it can no longer conclude that the activity will not present an unreasonable risk of injury to health or the environment, it will notify the submitter in writing that EPA is revoking its approval and state its reasons. In that event, the submitter must terminate the research and development activity within 48 hours of receipt of the notice in accordance with directions provided by EPA in the notice.

(b) *Evidence of unreasonable risk.* (1) If, after approval of a TERA under this

subpart, EPA determines that the proposed research and development activity will present an unreasonable risk of injury to health or the environment, EPA will notify the submitter in writing and state its reasons.

(2) In the notice, EPA may prescribe additional safeguards to address or reduce the risk, or may instruct the submitter to suspend the research and development activities.

(3) Within 48 hours, the submitter must implement the instructions contained in the notice. The submitter may then submit additional information or arguments concerning the matters raised by EPA and whether EPA should modify or revoke the approval of the TERA in accordance with paragraph (a)(2) of this section.

(4) EPA will consider the information and arguments in accordance with paragraph (a)(3) of this section.

(5) Following consideration of the information and arguments under paragraph (a)(3) of this section, if EPA notifies the submitter that the R&D activity must be suspended or terminted, the submitter may resume the activity only upon written notice from EPA that EPA has approved resumption of the activity. In approving resumption of an activity, EPA may prescribe additional conditions which must be followed by the submitter.

(c) *Modifications.* If, after approval of a TERA under this subpart, the submitter concludes that it is necessary to alter the conduct of the research and development activity in a manner which would result in the activity being different from that described in the TERA agreement and any conditions EPA prescribed in its approval, the submitter must inform the EPA contact for the TERA and may not modify the activity without the approval of EPA.

Subpart F—Exemptions for Test Marketing

§ 725.300 Scope and purpose.

(a) This subpart describes exemptions from the reporting requirements under subpart D of this part for test marketing activities involving microorganisms.

(b) In lieu of complying with subpart D of this part, persons described in § 725.305 may submit an application for a test marketing exemption (TME).

(c) Submission requirements specific for TME applications are described at § 725.350.

(d) Data requirements for TME applications are set forth in § 725.355.

(e) EPA review procedures specific for TMEs are set forth in § 725.370.

(f) Subparts A through C of this part apply to any submission under this subpart.

§ 725.305 Persons who may apply under this subpart.

A person identified in this section may apply for a test marketing exemption. EPA may grant the exemption if the person demonstrates that the microorganism will not present an unreasonable risk of injury to health or the environment as a result of the test marketing. A person may apply under this subpart for the following test marketing activities:

(a) A person who intends to manufacture or import for commercial purposes a new microorganism.

(b) A person who intends to manufacture, import, or process for commercial purposes a microorganism identified in subpart M of this part for a significant new use.

§ 725.350 Procedural requirements for this subpart.

General requirements for all submissions under this part are contained in subparts A through C of this part. In addition, the following requirements apply to applications submitted under this subpart:

(a) *Prenotice consultation.* EPA strongly suggests that for a TME, the applicant contact EPA for a prenotice consultation regarding eligibility for a TME.

(b) *When to submit a TME application.* Each person who is eligible to apply for a TME under this subpart must submit the application at least 45 calendar days before the person intends to commence the test marketing activity.

(c) *Recordkeeping.* Each person who is granted a TME must comply with the recordkeeping requirements of § 725.65. In addition, any person who obtains a

TME must retain documentation of compliance with any restrictions imposed by EPA when it grants the TME. This information must be retained for 3 years from the final date of manufacture or import under the exemption.

§ 725.355 Information to be included in the TME application.

(a) To review a TME application, EPA must have sufficient information to permit a reasoned evaluation of the health and environmental effects of the planned test marketing activity. The person seeking EPA approval must submit all information known to or reasonably ascertainable by the person on the microorganism and the test marketing activity, including information not listed in paragraphs (c), (d), and (e) of this section that the person believes will demonstrate that the microorganism will not present an unreasonable risk of injury to health or the environment as a result of the test marketing. The TME application must be in writing and must include at least the information described in paragraphs (b), (c), (d), and (e) of this section.

(b) When specific information is not submitted, an explanation of why such information is not available or not applicable must be included.

(c) Persons applying for a TME must submit the submitter identification and microorganism identity information required for MCANs in § 725.155(c), (d)(1), and (d)(2).

(d) Persons applying for a TME must submit phenotypic and ecological characteristics information required in § 725.155(d)(3) as it relates directly to the conditions of the proposed test marketing activity.

(e) Persons applying for a TME must also submit the following information about the proposed test marketing activity:

(1) *Proposed test marketing activity.* (i) The maximum quantity of the microorganism which the applicant will manufacture or import for test marketing.

(ii) The maximum number of persons who may be provided the microorganism during test marketing.

(iii) The maximum number of persons who may be exposed to the microorganism as a result of test marketing, including information regarding duration and route of such exposures.

(iv) A description of the test marketing activity, including its duration and how it can be distinguished from full-scale commercial production and research and development activities.

(2) *Health and environmental effects data.* All existing data regarding health and environmental effects of the microorganism must be reported in accordance with § 725.160.

§ 725.370 EPA review of the TME application.

General procedures for review of all submissions under this part are contained in §§ 725.28 through 725.60. In addition, the following procedures apply to EPA review of TME applications submitted under this subpart:

(a) No later than 45 days after EPA receives a TME, the Agency will either approve or deny the application.

(b) A submitter may only proceed with test marketing activities after receipt of EPA approval.

(c) In approving a TME application, EPA may impose any restrictions necessary to ensure that the microorganism will not present an unreasonable risk of injury to health and the environment as a result of test marketing.

Subpart G—General Exemptions for New Microorganisms

§ 725.400 Scope and purpose.

(a) This subpart describes exemptions from reporting under subpart D of this part, and from review under this part altogether, for manufacturing and importing of certain new microorganisms for commercial purposes.

(b) Recipient microorganisms eligible for the tiered exemption from review under this part are listed in § 725.420.

(c) Criteria for the introduced genetic material contained in the new microorganisms are described in § 725.421.

(d) Physical containment and control technologies are described in § 725.422.

(e) The conditions for the Tier I exemption are listed in § 725.424.

(f) In lieu of complying with subpart D of this part, persons using recipient microorganisms eligible for the tiered

exemption may submit a Tier II exemption request. The limited reporting requirements for the Tier II exemption, including data requirements, are described in §§ 725.450 and 725.455.

(g) EPA review procedures for the Tier II exemption are set forth in § 725.470.

(h) Subparts A through C of this part apply to any submission under this subpart.

§ 725.420 Recipient microorganisms.

The following recipient microorganisms are eligible for either exemption under this subpart:

(a) *Acetobacter aceti.*
(b) *Aspergillus niger.*
(c) *Aspergillus oryzae.*
(d) *Bacillus licheniformis.*
(e) *Bacillus subtilis.*
(f) *Clostridium acetobutylicum.*
(g) *Escherichia coli* K-12.
(h) *Penicillium roqueforti.*
(i) *Saccharomyces cerevisiae.*
(j) *Saccharomyces uvarum.*

§ 725.421 Introduced genetic material.

For a new microorganism to qualify for either exemption under this subpart, introduced genetic material must meet all of the criteria listed in this section.

(a) *Limited in size.* The introduced genetic material must consist only of the following:

(1) The structural gene(s) of interest.

(2) The regulatory sequences permitting the expression of solely the gene(s) of interest.

(3) Associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites.

(4) The nucleotide sequences needed for vector transfer.

(5) The nucleotide sequences needed for vector maintenance.

(b) *Well-characterized.* For introduced genetic material, well-characterized means that the following have been determined:

(1) The function of all of the products expressed from the structural gene(s).

(2) The function of sequences that participate in the regulation of expression of the structural gene(s).

(3) The presence or absence of associated nucleotide sequences and their associated functions, where associated nucleotide sequences are those sequences needed to move genetic material including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites.

(c) *Poorly mobilizable.* The ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient.

(d) *Free of certain sequences.* (1) The introduced genetic material must not contain a functional portion of any of the toxin-encoding sequences described in this paragraph (d).

(i) For the purposes of this section, a functional portion of a toxin-encoding sequence means any sequence which codes for a polypeptide that has one of the following effects:

(A) It directly or indirectly contributes to toxic effects in humans. Directly contributes to toxic effects in humans means those sequences encoding polypeptides that have direct toxicity to target cells. An example of a sequence which directly contributes to toxic effects in humans is one which encodes the portion of diphtheria toxin, listed in paragraph (d)(2) of this section, capable of interacting with elongation factor 2, leading to inhibition of protein synthesis in target respiratory, heart, kidney, and nerve tissues. Indirectly contributes to toxic effects in humans means a sequence whose encoded polypeptide is not directly toxic to target cells, yet still adversely affects humans. An example of a sequence which indirectly contributes to toxic effects is the sequence which encodes the portion of the botulinum toxin, listed in paragraph (d)(3) of this section, capable of blocking the release of acetylcholine from gangliosides. Botulinum toxin affects neuromuscular junctions by its blockage of acetylcholine release, leading to irreversible relaxation of muscles and respiratory arrest.

(B) It binds a toxin or toxin precursor to target human cells.

(C) It facilitates intracellular transport of a toxin in target human cells.

(ii) While these toxins are listed (with synonyms in parentheses) in paragraphs (d)(2) through (d)(7) of this section according to the source organism, it is use of the nucleotide sequences that encode the toxins that is being restricted and not the use of the source organisms. The source organisms are listed to provide specificity in identification of sequences whose use is restricted. Although similar or identical sequences may be isolated from organisms other than those listed below in paragraphs (d)(2) through (d)(7) of this section, these comparable toxin sequences, regardless of the organism from which they are derived, must not be included in the introduced genetic material.

(2) *Sequences for protein synthesis inhibitor.*

Sequence Source	Toxin Name
Corynebacterium diphtheriae & C. ulcerans	Diphtheria toxin
Pseudomonas aeruginosa	Exotoxin A
Shigella dysenteriae	Shigella toxin (Shiga toxin, Shigella dysentariae type I toxin, Vero cell toxin)
Abrus precatorius, seeds	Abrin
Ricinus communis, seeds	Ricin

(3) *Sequences for neurotoxins.*

Sequence Source	Toxin Name
Clostridium botulinum	Neurotoxins A, B, C1, D, E, F, G (Botulinum toxins, botulinal toxins)
Clostridium tetani	Tetanus toxin (tetanospasmin)
Proteus mirabilis	Neurotoxin
Staphylococcus aureus	Alpha toxin (alpha lysin)
Yersinia pestis	Murine toxin
Snake toxins	
Bungarus caeruleus	Caeruleotoxin
Bungarus multicinctus	Beta-bungarotoxin (phospholipase)
Crotalus spp.	Crotoxin (phospholipase)
Dendroaspis viridis	Neurotoxin
Naja naja varieties	Neurotoxin
Notechis scutatus	Notexin (phospholipase)
Oxyuranus scutellatus	Taipoxin
Invertebrate toxins	
Chironex fleckeri	Neurotoxin
Androctnus australis	Neurotoxin
Centruroides sculpturatus	Neurotoxin

(4) *Sequences for oxygen labile cytolysins.*

Sequence Source	Toxin Name
Bacillus alvei	Alveolysin
Bacillus cereus	Cereolysin
Bacillus laterosporus	Laterosporolysin
Bacillus thuringiensis	Thuringiolysin
Clostridium bifermentans	Lysin
Clostridium botulinum	Lysin
Clostridium caproicum	Lysin
Clostridium chauvoei	Delta-toxin
Clostridium histolyticum	Epsilon-toxin
Clostridium novyi	Gamma-toxin
Clostridium oedematiens	Delta-toxin
Clostridium perfringens	Theta-toxin (Perfringolysin)
Clostridium septicum	Delta-toxin
Clostridium sordellii	Lysin
Clostridium tetani	Tetanolysin
Listeria monocytogenes	Listeriolysin (A B)
Streptococcus pneumoniae	Pneumolysin
Streptococcus pyogene	Streptolysin O (SLO)

(5) *Sequences for toxins affecting membrane function.*

Sequence Source	Toxin Name
Bacillus anthracis	Edema factor (Factors I II); Lethal factor (Factors II III)
Bacillus cereus	Enterotoxin (diarrheagenic toxin, mouse lethal factor)
Bordetella pertussis	Adenylate cyclase (Heat-labile factor); Pertussigen (pertussis toxin, islet activating factor, histamine sensitizing factor, lymphocytosis promoting factor)
Clostridium botulinum	C2 toxin
Clostridium difficile	Enterotoxin (toxin A)
Clostridium perfringens	Beta-toxin; Delta-toxin
Escherichia coli & other Enterobacteriaceae spp.	Heat-labile enterotoxins (LT); Heat-stable enterotoxins (STa, ST1 subtypes ST1a ST1b; also STb, STII)
Legionella pneumophila	Cytolysin
Vibrio cholerae & Vibrio mimicus	Cholera toxin (choleragen)

(6) Sequences that affect membrane integrity.

Sequence Source	Toxin Name
Clostridium bifermentans & other *Clostridium spp*	Lecithinase
Clostridium perfringens	Alpha-toxin (phospholipase C, lecithinase); Enterotoxin
Corynebacterium pyogenes & other Corynebacterium spp.	Cytolysin (phospholipase C), Ovis toxin (sphingomyelinase D)
Staphylococcus aureus	Beta-lysin (beta toxin)

(7) *Sequences that are general cytotoxins.*

Sequence Source	Toxin Name
Adenia digitata	Modeccin
Aeromonas hydrophila	Aerolysin (beta-lysin, cytotoxic lysin)
Clostridium difficile	Cytotoxin (toxin B)
Clostridium perfringens	Beta-toxin; Epsilon-toxin; Kappa-toxin
Escherichia coli & other Enterobacteriaceae spp.	Cytotoxin (Shiga-like toxin, Vero cell toxin)
Pseudomonas aeruginosa	Proteases
Staphylococcus aureus	Gamma lysin (Gamma toxin); Enterotoxins (SEA, SEB, SEC, SED SEE); Pyrogenic exotoxins A B; Toxic shock syndrome toxins (TSST-1)

Sequence Source	Toxin Name
Staphylococcus aureus & *Pseudomonas aeruginosa*	Leucocidin (leukocidin, cytotoxin)
Streptococcus pyogenes	Streptolysin S (SLS); Erythrogenic toxins (scarlet fever toxins, pyrogenic exotoxins)
Yersinia enterocolitica	Heat-stable enterotoxins (ST)

§ 725.422 Physical containment and control technologies.

The manufacturer must meet all of the following criteria for physical containment and control technologies for any facility in which the new microorganism will be used for a Tier I exemption; these criteria also serve as guidance for a Tier II exemption.

(a) Use a structure that is designed and operated to contain the new microorganism.

(b) Control access to the structure.

(c) Provide written, published, and implemented procedures for the safety of personnel and control of hygiene.

(d) Use inactivation procedures demonstrated and documented to be effective against the new microorganism contained in liquid and solid wastes prior to disposal of the wastes. The inactivation procedures must reduce viable microbial populations by at least 6 logs in liquid and solid wastes.

(e) Use features known to be effective in minimizing viable microbial populations in aerosols and exhaust gases released from the structure, and document use of such features.

(f) Use systems for controlling dissemination of the new microorganism through other routes, and document use of such features.

(g) Have in place emergency clean-up procedures.

§ 725.424 Requirements for the Tier I exemption.

(a) *Conditions of exemption.* The manufacture or import of a new microorganism for commercial purposes is not subject to review under this part if all of the following conditions are met for all activities involving the new microorganism:

(1) The recipient microorganism is listed in and meets any requirements specified in § 725.420.

(2) The introduced genetic material meets the criteria under § 725.421.

(3) The physical containment and control technologies of any facility in which the microorganism will be manufactured, processed, or used meet the criteria under § 725.422.

(4) The manufacturer or importer submits a certification described in paragraph (b) of this section to EPA at least 10 days before commencing initial manufacture or import of a new microorganism derived from a recipient microorganism listed in § 725.420.

(5) The manufacturer or importer complies with the recordkeeping requirements of § 725.65 and maintains records for the initial and subsequent uses of the new microorganism that verify compliance with the following:

(i) The certifications made in paragraph (b) of this section.

(ii) All the eligibility criteria for the Tier I exemption including the criteria for the recipient microorganism, the introduced genetic material, the physical containment and control technologies.

(b) *Certification.* To be eligible for the Tier I exemption under this subpart, the manufacturer or importer must submit to EPA a document signed by a responsible company official containing the information listed in this paragraph.

(1) Name and address of manufacturer or importer.

(2) Date when manufacture or import is expected to begin.

(3) The identification (genus, species) of the recipient microorganism listed in § 725.420 which is being used to create the new microorganism which will be used under the conditions of the Tier I exemption.

(4) Certification of the following:

(i) Compliance with the introduced genetic material criteria described in § 725.421.

(ii) Compliance with the containment requirements described in § 725.422, including the provision in paragraph (a)(3) of this section.

(5) The site of waste disposal and the type of permits for disposal, the permit numbers and the institutions issuing the permits.

(6) The certification statement required in § 725.25(b). Certification of submission of test data is not required for the Tier I exemption.

§ 725.426 Applicability of the Tier I exemption.

The Tier I exemption under § 725.424 applies only to a manufacturer or importer of a new microorganism that certifies that the microorganism will be used in all cases in compliance with §§ 725.420, 725.421, and 725.422.

§ 725.428 Requirements for the Tier II exemption.

The manufacturer or importer of a new microorganism for commercial purposes may submit to EPA a Tier II exemption request in lieu of a MCAN under subpart D of this part if all of the following conditions are met:

(a) The recipient microorganism is listed in and meets any requirements specified in § 725.420.

(b) The introduced genetic material meets the criteria under § 725.421.

(c) Adequate physical containment and control technologies are used. The criteria listed under § 725.422 for physical containment and control technologies of facilities should be used as guidance to satisfy the Tier II exemption request data requirements listed at § 725.455(d). EPA will review proposed process and containment procedures as part of the submission for a Tier II exemption under this section.

§ 725.450 Procedural requirements for the Tier II exemption.

General requirements for all submissions under this part are contained in § 725.25. In addition, the following requirements apply to requests submitted under this subpart:

(a) *Prenotice consultation.* EPA strongly suggests that for a Tier II exemption, the submitter contact the Agency for a prenotice consultation regarding eligibility for the exemption.

(b) *When to submit the Tier II exemption request.* Each person who is eligible to submit a Tier II exemption request under this subpart must submit the request at least 45 calendar days before the person intends to commence manufacture or import.

(c) *Contents of the Tier II exemption request.* Each person who submits a request under this subpart must provide the information described in §§ 725.428 and 725.455, as well as information known to or reasonably ascertainable by the person that would permit EPA to determine that use of the microorganism, under the conditions specified in the request, will not present an unreasonable risk of injury to health or the environment.

(d) *Recordkeeping.* Each person who submits a request under this subpart must comply with the recordkeeping requirements of § 725.65. In addition, the submitter should maintain records which contain information that verifies compliance with the following:

(1) The certifications made in the request.

(2) All the eligibility criteria for the Tier II exemption request including the criteria for the recipient microorganism, the introduced genetic material, the physical containment and control technologies.

§ 725.455 Information to be included in the Tier II exemption request.

The submitter must indicate clearly that the submission is a Tier II exemption request for a microorganism instead of the MCAN under subpart D of this part and must submit the following information:

(a) *Submitter identification.* (1) The name and headquarters address of the submitter.

(2) The name, address, and office telephone number (including area code) of the principal technical contact representing the submitter.

(b) *Microorganism identity information.* (1) Identification (genus, species, and strain) of the recipient microorganism. Genus, species designation should be substantiated by a letter from a culture collection or a brief summary of the results of tests conducted for taxonomic identification.

(2) Type of genetic modification and the function of the introduced genetic material.

(3) Site of insertion.

(4) Certification of compliance with the introduced genetic material criteria described in § 725.421.

(c) *Production volume.* Production volume, including total liters per year, and the maximum cell concentration achieved during the production process.

(d) *Process and containment information.* (1) A description of the process including the following:

(i) Identity and location of the manufacturing site(s).

(ii) Process flow diagram illustrating the production process, including downstream separations, and indicating the containment envelope around the appropriate equipment.

(iii) Identities and quantities of feedstocks.

(iv) Sources and quantities of potential releases to both the workplace and environment, and a description of engineering controls, inactivation procedures, and other measures which will reduce worker exposure and environmental releases.

(v) A description of procedures which will be undertaken to prevent fugitive emissions, i.e. leak detection and repair program.

(vi) A description of procedures/safeguards to prevent and mitigate accidental releases to the workplace and the environment.

(2) Certification of those elements of the containment criteria described in § 725.422 with which the manufacturer is in compliance, including stating by number the elements with which the manufacturer is in full compliance.

(e) The site of waste disposal and the type of permits for disposal, the permit numbers and the institutions issuing the permits.

(f) The certification statement required in § 725.25(b). Certification of submission of test data is not required for the Tier II exemption.

§ 725.470 EPA review of the Tier II exemption request.

General procedures for review of all submissions under this part are contained in §§ 725.28 through 725.60. In addition, the following procedures apply to EPA review of Tier II exemption requests submitted under this subpart:

(a) *Length of the review period.* The review period for the request will be 45 days from the date the Document Control Officer for the Office of Pollution Prevention and Toxics receives a complete request, or the date EPA determines the request is complete under § 725.33, unless the Agency extends the review period for good cause under § 725.56.

(b) *Criteria for review.* EPA will review the request to determine that the new microorganism complies with § 725.428 and that its manufacture, processing, use, and disposal as described in the request will not present an unreasonable risk of injury to health or the environment.

(c) *EPA decision regarding the Tier II exemption request.* A decision concerning a request under this subpart will be made by the Administrator, or a designee.

(d) *Determination that the microorganism is ineligible for a Tier II review.* (1) EPA may determine that the manufacturer or importer is not eligible for Tier II review, because the microorganism does not meet the criteria under § 725.428 or the Administrator, or a designee, decides that there is insufficient information to determine that the conditions of manufacture, processing, use, or disposal of the microorganism as described in the request will not present an unreasonable risk to health or the environment.

(2) If the Agency makes this determination, the Administrator, or a designee will notify the manufacturer or importer by telephone, followed by a letter, that the request has been denied. The letter will explain reasons for the denial.

(3) If the request is denied, the manufacturer or importer may submit the information necessary to constitute a MCAN under subpart D of this part.

(e) *Approval or denial of the Tier II exemption request.* (1) No later than 45 days after EPA receives a request, the Agency will either approve or deny the request.

(2) In approving a request, EPA may impose any restrictions necessary to ensure that the microorganism will not present an unreasonable risk of injury to health and the environment as a result of general commercial use.

(f) EPA may seek to enjoin the manufacture or import of a microorganism in violation of this subpart, or act to seize any microorganism manufactured or imported in violation of this section or take other actions under the authority of sections 7 or 17 of the Act.

(g) A manufacturer or importer may only proceed after receipt of EPA approval.

Subparts H–K [Reserved]

Subpart L—Additional Procedures for Reporting on Significant New Uses of Microorganisms

§ 725.900 Scope and purpose.

(a) This subpart describes additional provisions governing submission of MCANs for microorganisms subject to significant new use rules identified in subpart M of this part.

(b) Manufacturers, importers, and processors described in § 725.105(c) must submit a MCAN under subpart D of this part for significant new uses of microorganisms described in subpart M of this part, unless they are excluded under §§ 725.910 or 725.912.

(c) Section 725.920 discusses exports and imports.

(d) Additional recordkeeping requirements specific to significant new uses of microorganisms are described in § 725.950.

(e) Section 725.975 describes how EPA will approve alternative means of complying with significant new use requirements designated in subpart M of this part.

(f) Expedited procedures for promulgating significant new use requirements under subpart M of this part for microorganisms subject to section 5(e) orders are discussed in §§ 725.980 and 725.984.

(g) This subpart L contains provisions governing submission and review of notices for the microorganisms and significant new uses identified in subpart M of this part. The provisions of this subpart L apply to the microorganisms and significant new uses identified in subpart M of this part, except to the extent that they are specifically modified or supplanted by specific requirements in subpart M of this part. In the event of a conflict between the provisions of this subpart L and the provisions of subpart M of this part, the provisions of subpart M of this part shall govern.

(h) The provisions of subparts A through F of this part also apply to subparts L and M of this part. For purposes of subparts L and M of this part, wherever the words "microorganism" or "new microorganism" appear in subparts A through F of this part, it shall mean the microorganism subject to subparts L and M of this part. In the event of a conflict between the provisions of subparts A through F and the provisions of subparts L and M of this part, the provisions of subparts L and M of this part shall govern.

§ 725.910 Persons excluded from reporting significant new uses.

(a) A person who intends to manufacture, import, or process a microorganism identified in subpart M of this part and who intends to distribute it in commerce is not required to submit a MCAN under subpart D of this part, if that person can document one or more of the following as to each recipient of the microorganism from that person:

(1) That the person has notified the recipient, in writing, of the specific section in subpart M of this part which identifies the microorganism and its designated significant new uses, or

(2) That the recipient has knowledge of the specific section in subpart M of this part which identifies the microorganism and its designated significant new uses, or

(3) That the recipient cannot undertake any significant new use described in the specific section in subpart M of this part.

(b) The manufacturer, importer, or processor described in paragraph (a) of this section must submit a MCAN under subpart D of this part, if such person has knowledge at the time of commercial distribution of the microorganism identified in the specific section in subpart M of this part that a recipient intends to engage in a designated significant new use of that microorganism without submitting a MCAN under this part.

(c) A person who processes a microorganism identified in a specific section in subpart M of this part for a significant new use of that microorganism is not required to submit a MCAN if that person can document each of the following:

(1) That the person does not know the specific microorganism identity of the microorganism being processed, and

(2) That the person is processing the microorganism without knowledge that the microorganism is identified in subpart M of this part.

(d)(1) If at any time after commencing distribution in commerce of a microorganism identified in a specific section in subpart M of this part, a person who manufactures, imports, or processes a microorganism described in subpart M of this part and distributes it in commerce has knowledge that a recipient of the microorganism is engaging in a significant new use of that microorganism designated in that section without submitting a MCAN under this part, the person is required to cease supplying the microorganism to that recipient and to submit a MCAN for that microorganism and significant new use, unless the person is able to document each of the following:

(i) That the person has notified the recipient and EPA enforcement authorities (at the address in paragraph (d)(1)(iii) of this section), in writing within 15 working days of the time the person develops knowledge that the recipient is engaging in a significant new use, that the recipient is engaging in a significant new use without submitting a MCAN.

(ii) That, within 15 working days of notifying the recipient as described in paragraph (d)(1)(i) of this section, the person received from the recipient, in writing, a statement of assurance that the recipient is aware of the terms of the applicable section in subpart M of this part and will not engage in the significant new use.

(iii) That the person has promptly provided EPA enforcement authorities with a copy of the recipient's statement of assurance described in paragraph (d)(1)(ii) of this section. The copy must be sent to the Director, Office of Compliance (2221A), Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460.

(2) If EPA notifies the manufacturer, importer, or processor that the recipient is engaging in a significant new use after providing the statement of assurance described in paragraph (d)(1)(ii) of this section and without submitting a MCAN under this part, the manufacturer, importer, or processor shall immediately cease distribution to that recipient until the manufacturer, importer, or processor or the recipient has submitted a MCAN under this part and the MCAN review period has ended.

(3) If, after receiving a statement of assurance from a recipient under paragraph (d)(1)(ii) of this section, a manufacturer, importer, or processor has knowledge that the recipient is engaging in a significant new use without submitting a MCAN under this part, the manufacturer, importer, or processor must immediately cease distributing the microorganism to that recipient and notify EPA enforcement authorities at the address identified in paragraph (d)(1)(iii) of this section. The manufacturer, importer, or processor may not resume distribution to that recipient until any one of the following has occurred:

(i) The manufacturer, importer, or processor has submitted a MCAN under this part and the MCAN review period has ended.

(ii) The recipient has submitted a MCAN under this part and the MCAN review period has ended.

(iii) The manufacturer, importer, or processor has received notice from EPA enforcement authorities that it may resume distribution to that recipient.

§ 725.912 Exemptions.

Persons identified in § 725.105(c) are not required to submit a MCAN under subpart D of this part for a microorganism identified in subpart M of this part, unless otherwise specified in a specific section in subpart M, if:

(a) The person submits a MCAN for the microorganism prior to the promulgation date of the section in subpart M of this part which identifies the microorganism, and the person receives written notification of compliance from EPA prior to the effective date of such section. The MCAN submitter must comply with any applicable requirement of section 5(b) of the Act. The MCAN must include the information and test data specified in section 5(d)(1) of the Act. For purposes of this exemption, the specific section in subpart M of this part which identifies the microorganism and §§ 725.3, 725.15,

725.65, 725.70, 725.75, 725.100, and 725.900 apply; after the effective date of the section in subpart M of this part which identifies the microorganism, §§ 725.105 and 725.910 apply and § 725.920 continues to apply. EPA will provide the MCAN submitter with written notification of compliance only if one of the following occurs:

(1) EPA is unable to make the finding that the activities described in the MCAN will or may present an unreasonable risk of injury to health or the environment under reasonably foreseeable circumstances, or

(2) EPA and the person negotiate a consent order under section 5(e) of the Act, such order to take effect on the effective date of the section in subpart M of this part which identifies the microorganism.

(b) The person is operating under the terms of a consent order issued under section 5(e) of the Act applicable to that person. If a provision of such section 5(e) order is inconsistent with a specific significant new use identified in subpart M of this part, abiding by the provision of the section 5(e) order exempts the person from submitting a MCAN for that specific significant new use.

§ 725.920 Exports and imports.

(a) *Exports.* Persons who intend to export a microorganism identified in subpart M of this part, or in any proposed rule which would amend subpart M of this part, are subject to the export notification provisions of section 12(b) of the Act. The regulations that interpret section 12(b) appear at part 707 of this chapter.

(b) *Imports.* Persons who import a substance identified in a specific section in subpart M of this part are subject to the import certification requirements under section 13 of the Act, which are codified at 19 CFR §§ 12.118 through 12.127 and 127.28(i). The EPA policy in support of the import certification requirements appears at part 707 of this chapter.

§ 725.950 Additional recordkeeping requirements.

Persons submitting a MCAN for a significant new use of a microorganism must comply with the recordkeeping requirements of § 725.65. In addition, the following requirements apply:

(a) At the time EPA adds a microorganism to subpart M of this part, EPA may specify appropriate recordkeeping requirements. Each manufacturer, importer, and processor of the microorganism shall maintain the records for 3 years from the date of their creation.

(b) The records required to be maintained under this section may include the following:

(1) Records documenting the information contained in the MCAN submitted to EPA.

(2) Records documenting the manufacture and importation volume of the microorganism and the corresponding dates of manufacture and import.

(3) Records documenting volumes of the microorganism purchased domestically by processors of the microorganism, names and addresses of suppliers and corresponding dates of purchase.

(4) Records documenting the names and addresses (including shipment destination address, if different) of all persons outside the site of manufacture or import to whom the manufacturer, importer, or processor directly sells or transfers the microorganism, the date of each sale or transfer, and the quantity of the microorganism sold or transferred on such date.

§ 725.975 EPA approval of alternative control measures.

(a) In certain sections of subpart M of this part, significant new uses for the identified microorganisms are described as the failure to establish and implement programs providing for the use of either: specific measures to control worker exposure to or release of microorganisms which are identified in such sections, or alternative measures to control worker exposure or environmental release which EPA has determined provide substantially the same degree of protection as the specified control measures. Persons who manufacture, import, or process a microorganism identified in such sections and who intend to employ alternative measures to control worker exposure or environmental release must submit a request to EPA for a determination of equivalency before commencing

manufacture, import, or processing involving the alternative control measures.

(b) A request for a determination of equivalency must be submitted in writing to the Office of Pollution Prevention and Toxics, Document Control Officer, 7407, 1200 Pennsylvania Ave., NW., Washington, DC 20460: ATTN: SNUR Equivalency Determination, and must contain:

(1) The name of the submitter.

(2) The specific identity of the microorganism.

(3) The citation for the specific section in subpart M of this part which pertains to the microorganism for which the request is being submitted.

(4) A detailed description of the activities involved.

(5) The specifications of the alternative worker exposure control measures or environmental release control measures.

(6) A detailed analysis explaining why such alternative control measures provide substantially the same degree of protection as the specific control measures identified in the specific section in subpart M of this part which pertains to the microorganism for which the request is being submitted.

(7) The data and information described in §§ 725.155 and 725.160. If such data and information have already been submitted to EPA's Office of Pollution Prevention and Toxics, the submitter need only document that it was previously submitted, to whom, and the date it was submitted.

(c) Requests for determinations of equivalency will be reviewed by EPA within 45 days. Determinations under this paragraph will be made by the Director, or a designee. Notice of the results of such determinations will be mailed to the submitter.

(d) If EPA notifies the submitter under paragraph (c) of this section that EPA has determined that the alternative control measures provide substantially the same degree of protection as the specified control measures identified in the specific section of subpart M of this part which pertains to the microorganism for which the request is being submitted, the submitter may commence manufacture, import, or processing in accordance with the specifications for alternative worker exposure control measures or environmental release control measures identified in the submitter's request, and may alter any corresponding notification to workers to reflect such alternative controls. Deviations from the activities described in the EPA notification constitute a significant new use and are subject to the requirements of this part.

§ 725.980 Expedited procedures for issuing significant new use rules for microorganisms subject to section 5(e) orders.

(a) *Selection of microorganisms.* (1) In accordance with the expedited process specified in this section, EPA will issue significant new use notification requirements for each new microorganism that, after MCAN review under subpart D of this part, becomes subject to a final order issued under section 5(e) of the Act, except for an order that prohibits manufacture and import of the microorganism, unless EPA determines that significant new use notification requirements are not needed for the microorganism.

(2) If EPA determines that significant new use notifications requirements are not needed for a microorganism that is subject to a final order issued under section 5(e) of the Act, EPA will issue a notice in the FEDERAL REGISTER explaining why the significant new use requirements are not needed.

(b) *Designation of requirements.* (1) The significant new use notification and other specific requirements will be based on and be consistent with the provisions included in the final order issued for the microorganism under section 5(e) of the Act. EPA may also designate additional activities as significant new uses which will be subject to notification.

(2) Significant new use requirements and other specific requirements designated under this section will be listed in subpart M of this part. For each microorganism, subpart M of this part will identify:

(i) The microorganism name.

(ii) The activities designated as significant new uses.

(iii) Other specific requirements applicable to the microorganism, including recordkeeping requirements or any other requirements included in the final section 5(e) order.

(c) *Procedures for issuing significant new use rules*—(1) *Possible processes.* EPA will issue significant new use rules (SNURs) under this section by one of the following three processes: direct final rulemaking, interim final rulemaking, or notice and comment rulemaking. EPA will use the direct final rulemaking process to issue significant new use rules unless it determines that, in a particular case, one of the other processes is more appropriate.

(2) *Notice in the* FEDERAL REGISTER. FEDERAL REGISTER documents issued to propose or establish significant new uses under this section will contain the following:

(i) The microorganism identity or, if its specific identity is claimed confidential, an appropriate generic microorganism name and an accession number assigned by EPA.

(ii) The MCAN number.

(iii) A summary of EPA's findings under section 5(e)(1)(A) of the Act for the final order issued under section 5(e).

(iv) Designation of the significant new uses subject to, or proposed to be subject to, notification and any other applicable requirements.

(v) Any modification of subpart L of this part applicable to the specific microorganism and significant new uses.

(vi) If the FEDERAL REGISTER document establishes a final rule, or notifies the public that a final rule will not be issued after public comment has been received, the document will describe comments received and EPA's response.

(3) *Direct final rulemaking.* (i) EPA will use direct final rulemaking to issue a significant new use rule, when specific requirements will be based on and be consistent with the provisions included in the final order issued for the microorganism under section 5(e) of the Act. EPA will issue a final rule in the FEDERAL REGISTER following its decision to develop a significant new use rule under this section for a specific new microorganism.

(ii) The FEDERAL REGISTER document will state that, unless written notice is received by EPA within 30 days of publication that someone wishes to submit adverse or critical comments, the rule will be effective 60 days from the date of publication. The written notice of intent to submit adverse or critical comments should state which SNUR(s) will be the subject of the adverse or critical comments, if several SNURs are established through the direct final rule. If notice is received within 30 days that someone wishes to submit adverse or critical comments, the section(s) of the direct final rule containing the SNUR(s) for which a notice of intent to comment was received will be withdrawn by EPA issuing a document in the final rule section of the FEDERAL REGISTER, and a proposal will be published in the proposed rule section of the FEDERAL REGISTER. The proposal will establish a 30-day comment period.

(iii) If EPA, having considered any timely comments submitted in response to the proposal, decides to establish notification requirements under this section, EPA will issue a final rule adding the microorganism to subpart M of this part and designating the significant new uses subject to notification.

(4) *Interim final rulemaking.* (i) EPA will use the interim final rulemaking procedure to issue a significant new use rule, when specific requirements will be based on and be consistent with the provisions included in the final order issued for the microorganism under section 5(e) of the Act. The Agency will issue an interim final rule in the FEDERAL REGISTER following its decision to develop a significant new use rule for a specific new microorganism. The document will state EPA's reasons for using the interim final rulemaking procedure.

(A) The significant new use rule will take effect on the date of publication.

(B) Persons will be given 30 days from the date of publication to submit comments.

(ii) Interim final rules issued under this section shall cease to be in effect

180 days after publication unless, within the 180-day period, EPA issues a final rule in the FEDERAL REGISTER responding to any written comments received during the 30-day comment period specified in paragraph (c)(4)(i)(B) of this section and promulgating final significant new use notification requirements and other requirements for the microorganism.

(5) *Notice and comment rulemaking.* (i) EPA will use a notice and comment procedure to issue a significant new use rule, when EPA is designating additional activities which are not provisions included in the final order issued for the microorganism under section 5(e) of the Act as significant new uses which will be subject to notification. EPA will issue a proposal in the FEDERAL REGISTER following its decision to develop a significant new use rule under this section for a specific new microorganism. Persons will be given 30 days to comment on whether EPA should establish notification requirements for the microorganism under this part.

(ii) If EPA, having considered any timely comments, decides to establish notification requirements under this section, EPA will issue a final rule adding the microorganism to subpart M of this part and designating the significant new uses subject to notification.

(d) *Schedule for issuing significant new use rules.* (1) Unless EPA determines that a significant new use rule should not be issued under this section, EPA will issue a proposed rule, a direct final rule, or an interim final rule within 180 days of receipt of a valid notice of commencement under §725.190.

(2) If EPA receives adverse or critical significant comments following publication of a proposed or interim final rule, EPA will either withdraw the rule or issue a final rule addressing the comments received.

§725.984 Modification or revocation of certain notification requirements.

(a) *Criteria for modification or revocation.* EPA may at any time modify or revoke significant new use notification requirements for a microorganism which has been added to subpart M of this part using the procedures of §725.980. Such action may be taken under this section if EPA makes one of the following determinations, unless other information shows that the requirements should be retained:

(1) Test data or other information obtained by EPA provide a reasonable basis for concluding that activities designated as significant new uses of the microorganism will not present an unreasonable risk of injury to health or the environment.

(2) EPA has promulgated a rule under section 4 or 6 of the Act, or EPA or another agency has taken action under another law, for the microorganism that eliminates the need for significant new use notification under section 5(a)(2) of the Act.

(3) EPA has received MCANs for some or all of the activities designated as significant new uses of the microorganism and, after reviewing such MCANs, concluded that there is no need to require additional notice from persons who propose to engage in identical or similar activities.

(4) EPA has examined new information, or has reexamined the test data or other information supporting its finding under section 5(e)(1)(A)(ii)(I) of the Act and has concluded that a rational basis no longer exists for the findings that activities involving the microorganism may present an unreasonable risk of injury to health or the environment required under section 5(e)(1)(A) of the Act.

(5) Certain activities involving the microorganism have been designated as significant new uses pending the completion of testing, and adequate test data developed in accordance with applicable procedures and criteria have been submitted to EPA.

(b) *Procedures for limitation or revocation.* Modification or revocation of significant new use notification requirements for a microorganism that has been added to subpart M of this part using the procedures described in §725.980 may occur either at EPA's initiative or in response to a written request.

(1) Any affected person may request modification or revocation of significant new use notification requirements for a microorganism that has been added to subpart M of this part using the procedures described in §725.980 by

writing to the Director, or a designee, and stating the basis for such request. The request must be accompanied by information sufficient to support the request. All requests should be sent to the TSCA Document Processing Center (7407), Room L–100, U.S. Environmental Protection Agency, 1200 Pennsylvania Ave., NW., Washington, DC 20460, ATTN: Request to amend SNUR.

(2) The Director, or a designee, will consider the request, make a determination whether to initiate rulemaking to modify the requirements, and notify the requester of that determination by certified letter. If the request is denied, the letter will explain why EPA has concluded that the significant new use notification requirements for that microorganism should remain in effect.

(3) If EPA concludes that significant new use notification requirements for a microorganism should be limited or revoked, EPA will propose the changes in a notice in the FEDERAL REGISTER, briefly describe the grounds for the action, and provide interested parties an opportunity to comment.

Subpart M—Significant New Uses for Specific Microorganisms

§ 725.1000 Scope.

This subpart identifies uses of microorganisms which EPA has determined to be significant new uses under the authority of section 5(a)(2) of the Toxic Substances Control Act.

§ 725.1075 Burkholderia cepacia complex.

(a) *Microorganism and significant new uses subject to reporting.* (1) The microorganisms identified as the *Burkholderia cepacia* complex defined as containing the following nine species, *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia ambifaria*, *Burkholderia pyrrocinia*, *Burkholderia cepacia* genomovar VIII (*Burkholderia anthina*), and *Burkholderia cepacia* genomovars III and VI are subject to reporting under this section for the significant new uses described in paragraph (a)(2) of this section.

(2) The significant new use is any use other than research and development in the degradation of chemicals via injection into subsurface groundwater.

(b) [Reserved]

[68 FR 35320, June 13, 2003]

PART 745—LEAD-BASED PAINT POISONING PREVENTION IN CERTAIN RESIDENTIAL STRUCTURES

Subparts A–C [Reserved]

Subpart D—Lead-Based Paint Hazards

Subpart E—Residential Property Renovation

Subpart F—Disclosure of Known Lead-Based Paint and/or Lead-Based Paint Hazards Upon Sale or Lease of Residential Property

Subparts G–K [Reserved]

Subpart L—Lead-Based Paint Activities

Saccharomyces cerevisiae Final Risk Assessment

ATTACHMENT I--FINAL RISK ASSESSMENT OF

SACCHAROMYCES CEREVISIAE

(February 1997)

I. INTRODUCTION

Saccharomyces cerevisiae has an extensive history of use in the area of food processing. Also known as Baker's Yeast or Brewer's Yeast, this organism has been used for centuries as leavening for bread and as a fermenter of alcoholic beverages. With a prolonged history of industrial applications, this yeast has been either the subject of or model for various studies in the principles of microbiology. Jacob Henle based his theories of disease transmission on studies of strains of Brewer's Yeast. Currently, S. cerevisiae is the subject of a major international effort to characterize a eucaryotic genome (Anderson, 1992).

History of Commercial Use and Products Subject to TSCA Jurisdiction

Saccharomyces cerevisiae, in addition to its use in food processing, is widely used for the production of macromolecular cellular components such as lipids, proteins including enzymes, and vitamins (Bigelis, 1985; Stewart and Russell, 1985).

The Food and Drug Administration rates Brewer's Yeast extract as Generally Recognized as Safe (FDA, 1986). Furthermore, the National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules (DHHS, 1986) considers S. cerevisiae a safe organism. Most experiments involving S. cerevisiae have been exempted from the NIH Guidelines based on an analysis of safety (see Appendix C-II of the NIH Guidelines). While alcoholic beverages, vitamins, and bread leavening are covered under the Federal Food, Drug and Cosmetic Act, the production of enzymes and other macromolecules may be subject to TSCA regulation. The abundance of information on S. cerevisiae, derived from its role in industry, has positioned it as a primary model for genetic studies and, by extension, as a strong candidate for genetic manipulation for TSCA applications (Dynamac, 1990).

II. IDENTIFICATION AND CLASSIFICATION

A. Taxonomy and Characterization

Saccharomyces cerevisiae is a yeast. The organism can exist either as a singlecelled organism or as pseudomycelia. The cells reproduce by multilateral budding. It produces from one to four ellipsoidal, smoothwalled ascospores. S. cerevisiae can be differentiated from other yeasts based on growth characteristics and physiological traits: principally the ability to ferment individual sugars. Clinical identification of yeast is conducted using commercially available diagnostic kits which classify the organism through analysis of the ability of the yeast to utilize distinct carbohydrates as sole sources of carbon (Buesching et al., 1979; Rosini et al., 1982). More recently, developments in systematics have led to the design of sophisticated techniques for classification, including gasliquid chromatography of lysed whole cells (Brondz and Olsen, 1979).

As a result of the application of newer techniques arising from innovative approaches, the taxonomy of Saccharomyces is subject to greater scrutiny. The initial classification was based principally on

morphological characteristics with specific physiological and biochemical traits used to differentiate between isolates with similar morphological traits. Using these criteria, there are as many as 18 species listed in the literature. In addition, what had been classified as one large heterogeneous species, S. cerevisiae, may, in the future, be divided into four distinct species based on DNA homology studies. The four species are S. cerevisiae, S. bayanus (also known as S. uvarum), S. pasteurianus (also known as S. carlsbergensis), and S. paradoxus. All four represent industrially important species. None of these organisms or other closely related species has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment.

Any assessment of Saccharomyces must take into consideration the malleability of the current classification. For this assessment of S. cerevisiae the reviews of the organism are based on the classification proposed by Van der Walt (1971).

B. Related Species of Concern

None of the above strains or other closely related species has been associated with pathogenicity toward humans or has been shown to have adverse effects on the environment.

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Colonization and Pathogenicity

S. cerevisiae is a commonly used industrial microorganism and is ubiquitous in nature, being present on fruits and vegetables. Industrial workers and the general public come into contact with S. cerevisiae on a daily basis through bothinhalation and ingestion (see section IV). Saccharomyces spp. are frequently recovered from the stools and throats of normally healthy individuals. This indicates that humans are in constant contact with these yeasts.

There are individuals who may ingest large quantities of S. cerevisiae every day, for example, people who take the yeast as part of a "health food" regimen. Therefore, studies were conducted to ascertain whether the ingestion of large numbers of these yeasts might result in either colonization, or colonization and secondary spread to other organs of the body. It was found that the installation of very large numbers of S. cerevisiae into the colons of animals would result in both colonization and passage of the yeasts to draining lymph nodes. It required up to 10^{10} S. cerevisiae in a single oral treatment to rats to achieve a detectable passage from the intestine to the lymph nodes (Wolochow et al., 1961). The concentrations of S. cerevisiae required were well beyond those that would be encountered through normal human daily exposure.

S. cerevisiae is not considered a pathogenic microorganism, but has been reported rarely as a cause of opportunistic infections. Eng et al. (1984) described five cases of such infections and reviewed the literature on eight other S. cerevisiae infections (also briefly reviewed by Walsh and Pizzo, 1988). All of the patients in the cases had underlying disease. Some of them had also received antibiotic therapy, thereby suppressing normal bacterial flora and allowing mycotic organisms to become established.

A low concern for the pathogenicity of S. cerevisiae is also illustrated by a series of surveys conducted at hospitals over the last several years. S. cerevisiae accounted for less than 1% of all yeast infections isolated at a cancer hospital and in most of the cases the organism was isolated from the respiratory system (Kiehn et al., 1980). At YaleNew Haven Hospital over the past five years, there have been 50 isolates of S. cerevisiae recovered from patients; however, most of the isolates were considered contaminants (Dynamac, 1991).

2. Toxin Production

There have been no reports of isolates of S. cerevisiae that produce toxins against either humans or animals. However, S. cerevisiae has been shown to produce toxins against other yeasts. These toxins, termed "killer toxins", are proteins or glycoproteins produced by a range of yeasts. The yeasts have been

genetically modified to alter activity and are used in industrial settings as a means of controlling contamination of fermentation systems by other yeasts (Sid et al., 1988).

3. Measure of the Degree of Virulence

A number of individual virulence factors have been identified as being associated with the ability of yeasts to cause disease. The principal virulence factors associated with yeasts appear to be phospholipase A and lysophospholipase. It is believed that these enzymes enhance the ability of the yeast to adhere to the cellwall surface and result in colonization as a first step in the infectious process. Nonpathogenic yeast had considerably lower phospholipase activities. Of a wide range of fungi assayed for phospholipase production, S. cerevisiae was found to have the lowest level of activity (BarrettBee et al., 1985). Therefore, based on the phospholipase virulence factor S. cerevisiae is considered a nonpathogenic yeast.

A second factor associated with virulence in yeast is the ability of a fungus to impair the host's immune capabilities. The cell walls of most fungi have the capacity to impede the immune response of the host. In a study to determine the overall pathogenicity of a number of yeasts used in industrial processes, animals exposed to both high levels of S. cerevisiae and cortisone demonstrated a greater ability of the fungus to colonize compared with those animals treated with only the yeast. However, the animals suffered no illeffects from exposure to S. cerevisiae (Holzschu et al., 1979). Therefore, this study suggests that even with the addition of high levels of an immunosuppressant agent, S. cerevisiae appears to be nonpathogenic.

4. Ability to Transfer Virulence Factor Genes

S. cerevisiae does not carry virulence factors to humans or animals. However, the species does carry linear, doublestranded plasmids which can be transmitted to other Saccharomyces. These plasmids carry genes that encode the "killer toxins" discussed above can be transferred from one Saccharomyces to another. Therefore, gene constructs involving the incorporation of traits using these linear plasmids should be considered to be nonstable.

5. Summary

In conclusion, S. cerevisiae is a organism which has an extensive history of safe use. Despite considerable use of the organism in research and the presence of S. cerevisiae in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the human had a debilitating condition. Factors associated with the virulence of yeasts (i.e., phospholipases) indicate that this organism is nonpathogenic. The organism has not been shown to produce toxins to humans.

B. Environmental Hazards

S. cerevisiae is ubiquitous in nature. It has been recovered from a variety of sites under varying ecological conditions. The organism is used in a variety of industrial scenarios. S. cerevisiae is commonly recovered from a variety of fresh fruits and vegetables, generally those fruits with high levels of fermentable sugars. However, it is not listed as the causative agent of food spoilage for fruits and vegetables (Phaff et al., 1966). The only adverse effect to the environment noted in the literature is the presence of the "killer toxins" which is active against other strains of Saccharomyces.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

S. cerevisiae is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986).

No data were available for assessing the release and survival specifically for fermentation facilities using S. cerevisiae. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected

from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, i.e., near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use areamonitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

S. cerevisiae is a normal inhabitant of soils and is widespread in nature. S. cerevisiae is able to take up a wide variety of sugars and amino acids. These traits enhance the organism's ability for long term survival. S. cerevisiae can be isolated from fruits and grains and other materials with a high concentration of carbohydrates (LaVeck, 1991).

2. Releases

Estimates of the number of S. cerevisiae organisms released during production are tabulated in Table 1 (Reilly, 1991). The uncontrolled/untreated scenario assumes no control features for the fermentor offgases, and no inactivation of the fermentation broth for the liquid and solid waste releases. The containment criteria required for the full exemption scenario assume the use of features or equipment that minimize the number of viable cells in the fermentor off-gases. They also assume inactivation procedures resulting in a validated 6log reduction of the number of viable microorganisms in the liquid and solid wastes relative to the maximum cell density of the fermentation broth.

TABLE 1. Estimated Number of Viable Saccharomyces cerevisiae

Organisms Released During Production

Uncontrolled/ Full

Release Media Untreated Exemption Release

(cfu/day) (cfu/day) (days/year)

Air Vents $2x10^8$ - 1x1011 $<2x10^8$ - 1x1011 350

Rotary Drum Filter 250 250 350

Surface Water $7x10^{12}$ $7x10^{6}$ 90

Soil/Landfill $7x10^{14}$ $7x10^{8}$ 90

Source: Reilly, 1991

These are "worstcase" estimates which assume that the maximum cell density in the fermentation broth for fungi is 10^{7} cfu/ml, with a fermentor size of 70,000 liters, and the separation efficiency for the rotary drum filter is 99 percent.

3. Air

Specific data which indicate the survivability of S. cerevisiae in the atmosphere after release are currently unavailable. Survival of vegetative cells during aerosolization is typically limited due to stresses such as shear forces, desiccation, temperature, and UV light exposure. As with naturally-occurring strains, human exposure may occur via inhalation as the organisms are dispersed in the atmosphere attached to dust particles, or lofted through mechanical or air disturbance.

Air releases from fermentor offgas could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from this source, the sector averaging form of the Gaussian algorithm described in Turner (1970) was used. For purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by controls/equipment for offgases, potential human inhalation dose rates are estimated to range from 3.0×10^{3} to 1.5×10^{6} cfu/year for the uncontrolled/untreated scenario and less than that for systems with full exemptions. It should be noted that these estimates represent hypothetical exposures under reasonable worst case conditions (Versar, 1992).

4. Water

The concentrations of S. cerevisiae in surface water were estimated using stream flow values for water bodies receivingprocess wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release data (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/l). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers facilities that have a NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10year period. The use of this methodology to estimate concentrations of S. cerevisiae in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process. Estimated concentrations of S. cerevisiae in surface water for the uncontrolled/untreated and the full exemption scenarios are tabulated in Table 2 (Versar, 1992).

TABLE 2. S. cerevisiae Concentrations in Surface Water

Receiving

Flow Stream Flow Organisms

(MLD*) (cfu/l)

Mean 7Q10	Mean 7Q10		
Uncontrolled/Untreated			
10th Percentile	156	5.60	4.5×10^{4} 1.25×10^{6}
50th Percentile	768	68.13	9.11×10^{3} 1.03×10^{5}
Full Exemption			
10th Percentile	156	5.60	4.5×10^{-2} 1.25×100^{0}
50th Percentile	768	68.13	9.11×10^{-3} 1.03×10^{-1}

*MLD = million liters per day

Source: Versar, 1992

5. Soil

Since soil is a natural habitat for S. cerevisiae, it would be expected to survive well in soil. These releases could result in human and environmental exposure (Versar, 1992). It iscurrently estimated that over one million tons of naturally-occurring yeast are produced annually during brewing and distilling practices (LaVeck, 1991).

6. Summary

Although direct monitoring data are unavailable, worst case estimates do not suggest high levels of exposure of S. cerevisiae to either workers or the public resulting from normal fermentation operations.

V. INTEGRATED RISK ASSESSMENT

A. Discussion

There is an extensive history of use of and exposure to S. cerevisiae with a very limited record of adverse effects to the environment or human health. Yeast has been used for centuries as a leavening for bread and fermenter of beer without records of virulence. S. cerevisiae is currently classified as a class 1 containment organism under the NIH Guidelines based largely on the extensive history of safe use.

Factors associated with the development of disease states in fungi have been reviewed. Data suggests that only with the ingestion of high levels of S. cerevisiae or with the use of immunosuppressants can S. cerevisiae colonize in the body. Even under those conditions, there were no noted adverse effects. In the few cases which S. cerevisiae was found in association with a disease state, the host was a debilitated individual, generally with an impaired immune system. In other cases the organism was recovered from an immunologically privileged site (i.e., respiratory tract). Many scientists believe that under appropriate conditions any microorganism could serve as an opportunistic pathogen. The cases noted in the above Human Health Assessment, where S. cerevisiae was found in association with a disease state, appear to be classic examples of opportunistic pathogenicity (see III.A.3).

The organism is not a plant or animal pathogen. Despite the fact that S. cerevisiae is ubiquitous in nature, it has not been found to be associated with disease conditions in plants or animals. The only adverse environmental condition that was noted is the production of "killer toxins" by some strains of the yeast. These toxins have a target range that is limited to susceptible yeasts. The toxins, proteins and glycoproteins, are not expected to have a broad environmental effect based largely on the anticipated short persistence of the toxins in soil orwater and by the limited target range. S. cerevisiae "killer toxin" has been used industrially to provide a level of protection against contamination by other yeasts in the fermentation beer.

The current taxonomy of Saccharomyces is under revision based on the development of alternative criteria. However, this should not have a major effect on the risk associated with closely related species. Saccharomyces, as a genus, present low risk to human health or the environment. Criteria used to differentiate between species are based on their ability to utilize specific carbohydrates without relevance to pathogenicity. Nonetheless, this risk assessment applies to those organisms that fall under the classical definition of S. cerevisiae as described by van der Walt (1971).

S. cerevisiae is a ubiquitous organism which, despite its broad exposure, has very limited reported incidence of adverse effects. The extensive history of use, the diversity of products currently produced by the organism, and the attention given this organism as a model for genetic studies collectively makes this organism a prime candidate for full exemption. The increased knowledge derived from the ongoing research should further enhance this organisms' biotechnological uses.

B. Recommendation

Saccharomyces cerevisiae is recommended for the tiered exemption.

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Corn

Related Topics

Background

The major feed grains are corn, sorghum, barley, and oats. Corn is the primary U.S. feed grain, accounting for more than 90 percent of total feed grain production and use.

- Around 80 million acres of land are planted to corn, with the majority of the crop grown in the Heartland region.
- Most of the crop is used as the main energy ingredient in livestock feed.
- Corn is also processed into a multitude of food and industrial products including starch, sweeteners, corn oil, beverage and industrial alcohol, and fuel ethanol.
- The United States is a major player in the world corn trade market, with approximately 20 percent of the corn crop exported to other countries.

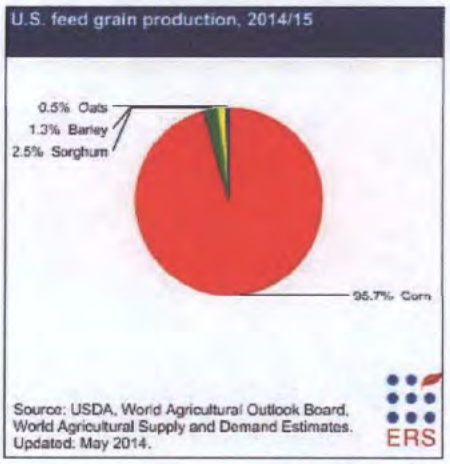

Chart data

Corn is grown in most U.S. States, but production is concentrated in the Heartland region (including Illinois, Iowa, Indiana, eastern portions of South Dakota and Nebraska, western Kentucky and Ohio, and the northern two-thirds of Missouri). Iowa and Illinois, the top corn-producing States, typically account for slightly more than one-third of the U.S. crop.

Because of provisions in the Federal Agriculture Improvement and Reform Act of 1996, corn acreage in the United States has increased from a government-mandated low of 60.2 million planted acres in 1983. The Act permitted

Related Data

- Agricultural Baseline Database
- Agricultural Outlook Statistical Indicators
- Commodity Costs and Returns
- Farm Program Atlas
- Feed Grains Database

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farmers to make their own crop planting decisions based on the most profitable crop for a given year. While the number of feed grain farms (those that produce corn, sorghum, barley, and/or oats) in the United States has declined in recent years, the acreage per corn farm has risen. Moreover, the number of large corn farms (with more than 500 acres) has increased over time, while the number of small corn farms (with less than 500 acres) has fallen.

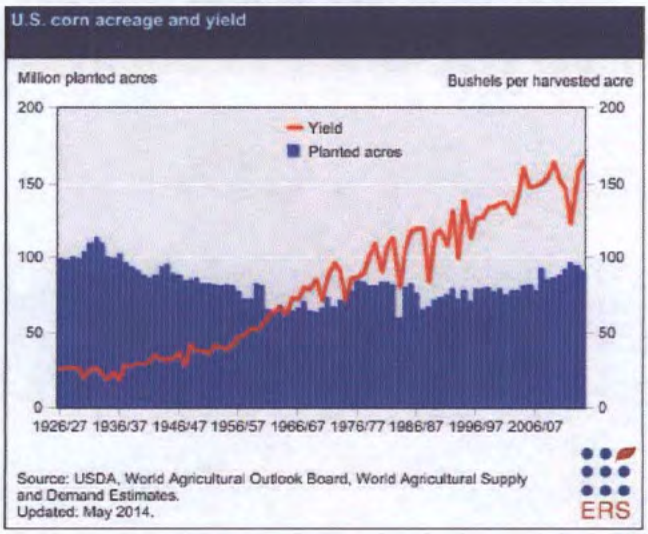

Chart data

Corn production has risen over time, as higher yields followed improvements in technology (seed varieties, fertilizers, pesticides, and machinery) and in production practices (reduced tillage, irrigation, crop rotations, and pest management systems).

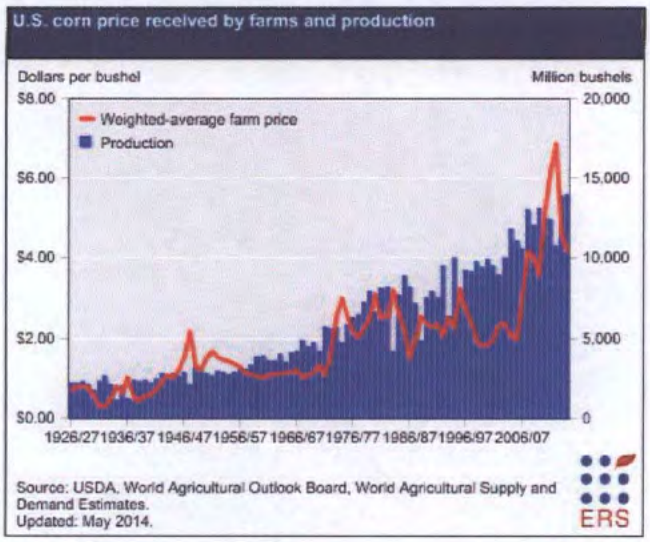

Chart data

Strong demand for ethanol production has resulted in higher corn prices and has provided incentives to increase corn acreage. In many cases, farmers have increased corn acreage by adjusting crop rotations between corn and soybeans, which has caused soybean plantings to decrease. Other sources of land for increased corn plantings include cropland used as pasture, reduced fallow, acreage returning to production from expiring Conservation Reserve Program contracts, and shifts from other crops, such as cotton.

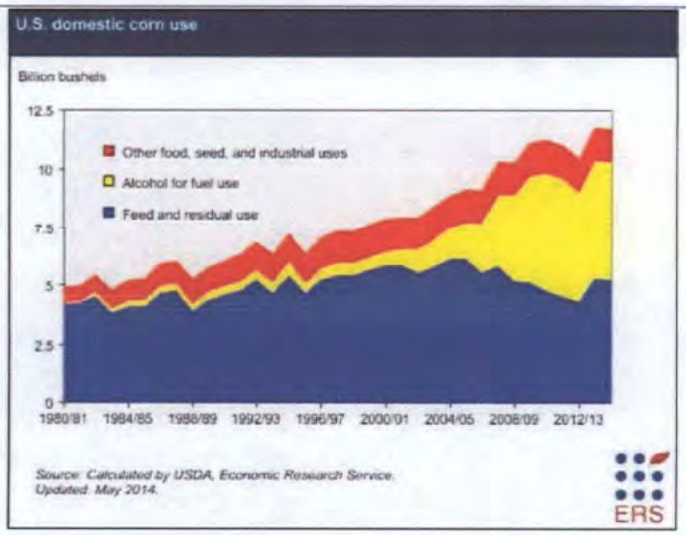

Chart data

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Corn has food, feed, and industrial uses. It is a major component of livestock feed. Feed use, a derived demand, is closely related to the number of animals (cattle, hogs, and poultry) that are fed corn. The amount of corn used for feed also depends on the crop's supply and price, the amount of supplemental ingredients used in feed rations, and the supplies and prices of competing ingredients.

As ethanol production increases, the supply of ethanol coproducts will also increase. Both the dry-milling and wet-milling methods of producing ethanol generate a variety of economically valuable coproducts, the most prominent of which is distillers dried grains with solubles (DDGs), which can be used as a feed ingredient for livestock. Each 56-pound bushel of corn used in dry-mill ethanol production generates about 17.4 pounds of DDGs. In the United States, cattle (both dairy and beef) have been the primary users of DDGS as livestock feed, but larger quantities of DDGs are making their way into the feed rations of hogs and poultry.

Corn is also processed for human consumption and other industrial uses. Food, seed, and industrial uses (FSI) of corn account for about one-third of domestic utilization. During processing, corn is either wet or dry milled depending on the desired end products:

- Wet millers process corn into high-fructose corn syrup (HFCS), glucose and dextrose, starch, corn oil, beverage alcohol, industrial alcohol, and fuel ethanol.
- Dry millers process corn into flakes for cereal, corn flour, corn grits, corn meal, and brewers grits for beer production.

The market for food made from corn has grown in recent years with the expanding Latin American population in the United States. In the future, food uses for corn are expected to expand at the rate of population growth. Research is continuing to expand the various industrial uses for corn and corn byproducts.

Farmers may be eligible to receive government payments that support or protect their income. These include production flexibility contract payments, marketing loans, disaster aid, conservation payments, and crop insurance. (See the Policy page for more information.) Government programs have been instrumental in the development of the HFCS and fuel alcohol markets. Import fees, duties, and import quotas on sugar have made HFCS an economical alternative. Recent Federal environmental laws have paved the way for greater use of corn in fuel alcohol production.

Corn is the largest component of global coarse grain trade (including corn, sorghum, barley, oats, rye, millet, and mixed grains), generally accounting for about two-thirds of the volume over the past decade. The United States is the world's largest corn producer and currently exports about one-fifth of annual production. (See the Trade page for more information.)

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Food and Agriculture Organization
of the United Nations

World Health Organization

Evaluation of Allergenicity of Genetically Modified Foods

Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology
22 – 25 January 2001

Food and Agriculture Organization of the United Nations (FAO)
Rome, Italy

The opinions expressed in this report are those of the participants at the Consultation and do not imply any opinion on the part of FAO and WHO

CONTENTS

1. Introduction

A Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology was held at the Headquarters of the Food and Agriculture Organization of the United Nations (FAO) in Rome from 22 to 25 January 2001. The Consultation was a follow-up of the Joint FAO/WHO Consultation held in Geneva, Switzerland from 29 May to 2 June 2000 and focused on the question of allergenicity of genetically modified foods. A total of 28 experts, including authors of discussion papers, participated in the Consultation. The complete list of participants is given in Annex 1.

Mr Jacque Vercueil, Director of the Agriculture and Economic Development Analysis Division, Economic and Social Department of FAO, opened the Consultation on behalf of the Directors-General of the World Health Organization (WHO) and FAO. In his statement, Mr Vercueil indicated that allergenicity was one of the most frequently asked questions in connection with the safety of genetically modified foods. It was urgently needed to establish a reliable methodology to assess the allergenicity of new foods produced by the recombinant DNA technique. Applying appropriate risk management measures could reduce the risk of allergenicity of genetically modified foods.

The Consultation elected Dr Dean Metcalfe as Chairperson and Dr Harris Steinman as Vice-Chairperson. Dr Steve Taylor was elected as Rapporteur. The Consultation agreed to base its discussion on the decision tree adapted by the previous FAO/WHO Consultation in 2000 (Annex 3). The Consultation decided to form two working groups to draft the report and to delegate the working groups to elect their chairpersons and rappporteurs: the first working group, considering mainly products created with genes obtained from known allergenic sources (the left-hand side of the existing decision tree, Annex 3) and post-market surveillance, decided that Dr Carsten Bindslev-Jensen be its Chairman and Dr David Hill be its Rapporteur and the second working group, considering mainly products created with genes obtained from sources with no history of allergenicity (the right-hand side of the existing decision tree, Annex 3), decided that Dr Rob Aalberse be its Chairperson and Dr Ricki Helm be its Rapporteur, respectively. The list of working documents is reproduced in Annex 2 to this report. The report entitled "Assessment of Scientific Information Concerning StarLink Corn" (EPA, 2000) was also introduced as an actual case where methodologies under discussion by the Consultation had been applied.

The Consultation further noted the specific questions in documents Biotech 01/02, submitted by the Joint FAO/WHO Secretariat of the Consultation.

2. Background

In 1990 and 1996 FAO and WHO organised joint expert consultations to consider the safety and nutritional aspects of genetically modified foods. The 1990 Consultation regarded biotechnology as a continuum, embracing traditional breeding techniques and modern techniques based on recombinant DNA technologies and concluded that foods from modern biotechnology were inherently not less safe than those from traditional biotechnology (WHO, 1991). The 1996 Consultation recommended that substantial equivalence be an important component in the safety assessment of foods and food ingredients derived from genetically modified plants intended for human consumption (FAO, 1996). The Codex Alimentarius Commission and its relevant subsidiary bodies had reflected the results of the both consultations.

Recognizing the rising concern among the world population about the safety and nutritional aspects of foods derived from biotechnology, the Codex Alimentarius Commission,

at its 23rd Session in 1999, decided to establish an *Ad Hoc* Intergovernmental Task Force on Foods Derived from Biotechnology to develop standards, guidelines or recommendations, as appropriate, for foods derived from biotechnology or traits introduced into foods by biotechnology. The first meeting of the Task Force was held in Japan in March 2000. FAO and WHO expressed their intention to organize a series of scientific expert consultations to support the work of the Task Force.

In June 2000, a Joint FAO/WHO Consultation on Foods Derived from Biotechnology was held in Geneva (WHO, 2000). It addressed the overall safety aspects of foods derived from genetically modified plants and focused on the applicability of substantial equivalence as a general guidance for scientific risk assessment. This Consultation identified specific areas on which further expert consultation was needed and recommended that FAO/WHO should convene an expert consultation on the assessment of allergenicity of genetically modified foods and the novel proteins contained therein as a matter of priority.

The 2000 Consultation adapted a decision-tree (Annex 3) for the evaluation of allergenicity of novel proteins introduced into genetically modified foods. It agreed that the reliability of the risk assessment procedures for allergenicity of genetically modified foods using the decision-tree approach should be further enhanced, including the consideration of additional criteria.

3. Scope

The Consultation was convened to provide FAO, WHO and their Member States with scientific advice in relation to the assessment of allergenicity in genetically modified foods. This would cover in particular:

- General consideration of allergenicity of genetically modified foods
  - consideration of allergenicity specifically relevant to genetically modified foods
- Consideration of the decision-tree approach
  - consideration and possible revision of the decision-tree for the assessment of allergenicity of genetically modified foods developed preliminarily by the June 2000 Joint FAO/WHO Consultation on Foods Derived from Biotechnology.
  - development of standardised procedures for consideration of the use of individual criteria used in the decision tree, with a view to a harmonised application of the decision-tree
  - consideration of the possibility of post market surveillance for inclusion in the decision-tree and technologies supporting the implementation of post market surveillance.
- Specific questions arising in relation to the assessment of allergenicity of genetically modified foods
  - use of databases in the assessment of allergenicity of genetically modified foods
  - use of animal testing
  - other related issues

4. Overview of Food Allergies

Food allergies are adverse reactions to an otherwise harmless food or food component that involves an abnormal response of the body's immune system to specific protein(s) in foods. True food allergies may involve several types of immunological responses (Sampson and Burks, 1996). The most common type of food allergy is mediated by allergen-specific immunoglobulin E (IgE) antibodies[1]. IgE-mediated reactions are known as immediate hypersensitivity reactions because symptoms occur within minutes to a few hours after ingestion of the offending food. IgE-mediated reactions may occur to pollens, mould spores, animal danders, insect venoms and other environmental stimuli as well as foods. IgE-mediated reactions affect perhaps 10-25% of the population in developed countries (Mekori, 1996), although food allergies represent a small fraction of all allergic diseases. Infants and young children are more commonly affected by IgE-mediated food allergies than adults; the prevalence among infants under the age of 3 may be as high as 5-8% (Bock, 1987; Sampson, 1990a; European Commission, 1998).

True food allergies also encompass delayed hypersensitivity reactions whose mechanisms are less clear. Such reactions include cell-mediated reactions that involve sensitised lymphocytes in tissues rather than antibodies (Sampson, 1990b). In cell-mediated reactions, the onset of symptoms occurs more than 8 hours after ingestion of the offending food. The overall prevalence of food-induced, cell-mediated reactions remains uncertain (Burks and Sampson, 1993), but these reactions are well documented in infants. Delayed, food-induced enteropathy has been observed in infants on exposure to milk, soybeans, and less frequently, other proteins. The most common cell-mediated hypersensitivity reaction affecting all age groups of the population is celiac disease, also known as gluten-sensitive enteropathy. Celiac disease affects 1 in every 300 to 3000 individuals in the population depending upon the specific geographic region.

Food allergies are caused by a wide variety of foods. The Codex Committee on Food Labelling established, after considerable debate, a list of the most common allergenic foods associated with IgE-mediated reactions on a worldwide basis that includes peanuts, soybeans, milk, eggs, fish, crustacea, wheat, and tree nuts. This list was presented to the Codex Alimentarius Commission and adopted in 1999 at its 23rd Session. These commonly allergenic foods account for over 90% of all moderate to severe allergic reactions to foods, although an extensive literature search has revealed more that 160 foods associated with sporadic allergic reactions (Hefle et al., 1996). Theoretically, any food that contains protein would be capable of eliciting an allergic reaction, although foods vary widely in their likelihood of provoking allergic sensitisation. In addition to the Codex list, allergic reactions to fresh fruits and vegetables, associated with the oral allergy syndrome (OAS), are also rather common (Ortolani et al., 1988). These foods are not included in the Codex list. The symptoms are typically mild and mostly confined to the oropharyngeal region. Some of the most significant allergens from these foods are unstable to heating and digestion. However, OAS in patients allergic to fruits and vegetables may, in some individuals, be followed by a systemic reaction (Ballmer-Weber et al., 2000). The list established by the Codex Committee on Food Labelling also includes gluten-containing cereals (wheat, rye, barley, oats and spelt) that are implicated in the aetiology of gluten-sensitive enteropathy.

In IgE-mediated food allergies, exposure to a specific food and the proteins contained therein elicits the development of food allergen-specific IgE antibodies. These IgE antibodies

[1] IgE, or immunoglobulin E, is a protein antibody that recognizes an allergen. It circulates in the blood, and becomes fixed on the surfaces of specific cells (basophils and mast cells). When IgE on the cell surface binds to allergen, this triggers the release of chemical mediators that provoke the symptoms associated with allergic reactions.

attach to the surfaces of mast cells and basophils, thus sensitising the individual to react upon subsequent exposure to the specific food. Thus, to become sensitised, individuals must first be exposed to the food in question. Some food proteins are more likely than others to elicit allergic sensitisation. Very limited information exists on the levels of exposure to a food that are minimally necessary to elicit allergic sensitisation in susceptible individuals. However, infants are much more likely to be sensitised than adults and are possibly sensitised to comparatively low levels of exposure to the offending food. Subsequent exposure of a sensitised individual to the offending food will likely elicit an allergic reaction. The allergen cross-links IgE antibodies on the surfaces of mast cells or basophils triggering the release of various mediators of the allergic reaction. These mediators are released into the tissues and blood, interacting with various receptors that provoke the symptoms characteristic of allergic reactions. The amount of exposure to an ingested, allergenic food protein needed to elicit a discernable reaction in already sensitised and highly sensitive individuals is not precisely known but appears to be in the microgram to low milligram range.

The manifestations of IgE-mediated food allergies range from mild to severe to life-threatening events. Individuals display different thresholds for elicitation of a reaction following ingestion of the offending food. However, the most sensitive food-allergic individuals will experience reactions from exposure to microgram to low milligram quantities or perhaps less of the offending food (limited studies have been conducted on threshold doses so the lowest-observed adverse effect level cannot be deduced precisely for any given allergenic food). Severe reactions can take place after intake of minute amounts of the offending food, and a safe threshold level below which reactions will not occur has not been defined.

Gluten-sensitive enteropathy or celiac disease is a T cell-mediated immunological response triggered by gluten (gliadin) which affects genetically disposed individuals. The active phase of the disease consists of an inflammatory process in the small intestine leading to malabsorption with body wasting, anaemia, diarrhoea, and bone pain along with other symptoms. The disease demands lifelong avoidance of gluten from wheat, rye, barley, and related cereals.

Celiac disease and other enteropathies, although recognized by this Consultation as important medical conditions, were not included in the assessment strategies considered by this Consultation.

Both IgE-mediated food allergies and non-IgE-mediated reactions are treated with specific avoidance diets. Since in both cases, the threshold dose is low and not precisely defined, affected individuals can experience difficulties in the adherence to the avoidance diets.

Almost all food allergens are proteins, although the possibility exists that other food components may act as haptens[2]. While some food allergens have been identified and characterized, many others remain unknown. Many of the known food allergens fall into certain classes of proteins which may aid in the identification of unknown allergens from other sources. Similarly, prolamin proteins from wheat, rye, barley, etc. are involved in the elicitation of gluten-sensitive enteropathy. While the crops from which staple foods are derived contain thousands of different proteins, relatively few are allergenic. The distribution of these proteins varies in different parts of the plant and can be influenced by environmental factors such as climate and disease stress.

Conventional breeding introduces additional protein diversity into the food supply. However, variations in the protein composition of our diets brought about through conventional

[2] Haptens are small molecules, which may interact with body proteins or food proteins and cause these proteins to become allergenic.

crop improvement practices have had little, if any, effect on the allergenic potential of our major foods. In contrast, altered dietary preferences and changes in food manufacturing and food formulation practices can have significant implications for the development of food allergies. For example, allergy to peanut (groundnut) occurs at a significant frequency in North America and Western Europe but not in other countries where peanuts are less commonly eaten. Also, recent food introductions such as kiwi fruit have proven to be additional sources of food allergens. With respect to food formulations, the wider distribution of certain ethnic foods, such as those containing sesame seeds, may contribute to increases in allergic sensitivity to certain foods. These observations provide confidence that there are not a large number of potential allergens in the food supply, but show that new allergenic foods are sometimes introduced into the marketplace.

Because of the above, a clear need exists to pay particular attention to allergenicity when assessing the safety of foods produced through genetic modification. In the assessment of the allergenicity of genetically modified foods, the characteristics of the novel gene products (proteins) must be evaluated in light of their similarities to known food and environmental allergens. In addition, if an examination of the genetically modified food in comparison to its conventional counterpart reveals the presence of any unintended, new proteins resulting from the transformation events, these unintended, new proteins should also be assessed for their possible allergenicity using a similar approach.

5. Decision Tree Approach to the Evaluation of the Allergenicity of Genetically Modified Foods

5.1. Introduction

In 1996, the International Food Biotechnology Council and the Allergy and Immunology Institute of the International Life Sciences Institute (IFBC/ILSI) presented a decision-tree approach to the evaluation of the potential allergenicity of the novel gene products (proteins) in genetically modified foods (Metcalfe et al., 1996). This allergy assessment strategy has been widely adopted by the agricultural biotechnology industry. It is a strategy which focuses on the source of the gene, the sequence homology of the newly introduced protein to known allergens, the immunochemical binding of the newly introduced protein with IgE from the blood serum of individuals with known allergies to the transferred genetic material, and the physicochemical properties of the newly introduced protein (Metcalfe et al., 1996; Taylor, 1997).

In the 1996 Joint FAO/WHO Consultation on Biotechnology and Food Safety, the issue of allergenicity of genetically modified foods was specifically addressed for the first time. An assessment approach similar to that developed by IFBC/ILSI was advocated that included the following criteria: source of the transferred genetic material, molecular weight, sequence homology, heat and processing stability, effect of pH and/or gastric juices (digestive stability), and prevalence in foods. The 1996 Consultation concluded that "a rational scientific approach to the assessment of the allergenicity of genetically modified organisms can and should be undertaken" as part of the overall safety assessment approach. Furthermore, the 1996 Consultation made several recommendations relative to allergenicity of genetically modified foods:

- The transfer of genes from commonly allergenic foods should be discouraged unless it is documented that the gene transferred does not code for an allergen.

- Foods found to contain an allergen transferred from the organism which provided the DNA should not be considered for marketing approval unless such products can be clearly identified in the marketplace and this identity will not be lost through distribution and processing. Further, that labelling approaches may not be practical in these situations, and that particular problems exist for consumers who cannot read, or who may not be provided with labels.
- Involved organizations should consider the appropriateness of, and/or actions to take, in respect to foods containing new protein(s) that are determined to have the characteristics of an allergen, even though no patient population is known to exist which has an allergy to this gene product.
- The identification of food allergens and the characteristics of these allergens that define their immunogenicity be encouraged.

In the 2000 Joint FAO/WHO Consultation on Safety Aspects of Genetically Modified Foods of Plant Origin, the issue of the allergenicity of genetically modified foods was specifically addressed again. The IFBC/ILSI decision-tree approach was adapted, with minor changes, for the evaluation of novel proteins introduced into genetically modified foods (Annex 3). The said Consultation concluded "that if a genetically modified food contains the product of a gene from a source with known allergenic effects, the gene product should be assumed to be allergenic unless proven otherwise. The transfer of genes from commonly allergenic foods should be discouraged unless it can be documented that the gene transferred does not code for an allergen. The novel proteins introduced into genetically modified food should be evaluated for allergenicity on the basis of the decision-tree shown in Annex 3." The 2000 Consultation noted that the IFBC/ILSI decision tree as adapted by FAO/WHO in Annex 3 had received some criticism related to certain of the criteria involved in the decision tree. The 2000 Consultation further concluded, "additional criteria should be considered for the addition to the decision-tree approach when the source of the genetic material is not known to be allergenic. The level and site of expression of the novel protein and the functional properties of the novel protein would be two such criteria."

The 2000 Joint FAO/WHO Consultation recommended that "WHO/FAO should be encouraged to convene an Expert Consultation on the assessment of the allergenicity of genetically modified foods and the novel proteins contained therein. The Consultation should focus on the development of an improved decision-tree approach for the assessment of the allergenicity of genetically modified foods and on the standardization/validation of specific criteria, such as optimal methods for assessment of digestive stability." With this background, the current consultation undertook efforts to develop an improved decision-tree approach using as a start, the existing IFBC/ILSI decision tree as adapted by the 2000 FAO/WHO Consultation (Annex 3) .

5.2. The FAO/WHO 2001 Decision Tree

After consideration of the current status of scientific information and extensive discussion, the Consultation developed a new decision tree (Annex 4) that will be referred to throughout the remainder of this report as the FAO/WHO 2001 decision tree. This new decision tree builds upon previous approaches to examining allergenicity but also encompasses several additional strategies.

5.3. Food containing a gene derived from a source known to be allergenic

When the expressed protein comes from a source known to be allergenic, the analysis presented in the FAO/WHO 2001 decision tree focuses on both sequence homology and subsequent assessment of potential allergenicity of the expressed protein with sera of patients allergic to the source material (Annex 4). Sequence homology is the initial step to be performed. Criteria for a positive outcome in the analysis of sequence homology are reviewed in Section 6.1. When sequence homology to a known allergen is demonstrated, the product is considered allergenic, and no further testing is typically undertaken. If no sequence homology to a known allergen is demonstrated, specific serum screening for the expressed protein is undertaken. These investigations focus on assessment of the possible allergenicity of the expressed protein using sera from patients allergic to the source material (Section 6.2). These patients should be carefully defined according to international guidelines. If the patients donating sera have a low level of sensitisation, the usefulness of those sera in exhibiting reactivity to the expressed protein may be compromised. Therefore, it is suggested to include only patients with a level of sensitisation to the allergen source of more than 10 kIU/L of specific IgE.

In contrast to previous decision-tree strategies, the FAO/WHO 2001 decision tree makes no distinction between commonly and less commonly allergenic source materials with respect to specific serum screening. Thus, specific serum screening is undertaken irrespective of the relative frequency of allergy to the source material in question, provided sera are available (Section 6.2). Insufficient information exists in the literature supporting an increased risk of a severe reaction for patients with hypersensitivity to commonly allergenic foods as opposed to less commonly allergenic foods.

The degree of confidence in the results of the specific serum screening will depend upon the number of sera that are available for analysis. To achieve 95 % certainty that a major allergen (a major allergen is defined as one to which more than 50 % of individuals sensitive to that substance react in IgE-specific immunoassays) from the source material has not been transferred, a negative result must be obtained with at least 6 relevant sera. To achieve 99 % certainty that a major allergen from the source material has not been transferred, a negative result must be obtained with at least 8 relevant sera. To achieve 99.9 % certainty that a major allergen from the source material has not been transferred, a negative result must be obtained with at least 14 relevant sera. Furthermore, by using 17 relevant test sera, a 95 % probability exists of detecting a minor allergen (a minor allergen is defined as one to which less than 50 % of individuals sensitive to that substance react in IgE-specific immunoassays) from the source to which at least 20 % of the affected population are reactive. By using 24 relevant sera, a 99 % probability exists of detecting a minor allergen from the source to which at least 20 % of the affected population are reactive. An argument can be made for using fewer sera if relevant sera are not available, but this modified approach carries the risk of a false negative outcome. However, the use of larger numbers of sera is advocated, whenever possible, to increase the confidence associated with negative immunoassay results as described above. The Consultation also recognizes that the use of a smaller number of very well documented, high quality sera may be preferable to the use of larger numbers of lesser-quality sera. The in vitro method applied should be a validated assay measuring specific IgE (Section 6.2).

Any positive outcome defines the product as likely allergenic, and will normally lead to discontinuation of product development. A negative outcome of the specific serum screening prompts further analysis using targeted serum screening (Section 6.3), pepsin resistance (Section 6.4) and animal models (Section 6.5) (see Annex 4). Additionally, in vivo/ex vivo[3] testing in

[3] "in vivo (using allergic human subjects)/ex vivo (using cells or tissue culture from allergic human subjects)

allergic patients may also be appropriate in circumstances where confirmation of positive results in the specific serum screening is wished; or where a negative outcome of appropriate in vivo/ex vivo testing would be more convincing than a positive outcome of the specific serum screening provided that well documented allergic subjects were used in the in vivo/ex vivo testing. The ex vivo/in vivo methods include skin prick testing (Bruijnzeel-Koomen et al, 1995), basophil histamine release (Bindslev-Jensen and Poulsen, 1996) and oral challenge (Bock et al, 1988; Bruijnzeel-Koomen et al, 1995). It is anticipated that these procedures will require approval from Ethics Committees (Internal Review Boards). Therefore, the FAO/WHO 2001 decision tree does not include human in vivo testing as a mandatory tool, but in vivo testing may be considered in selected cases.

An equivocal outcome of the specific serum screening would lead to further analysis using targeted serum screening, pepsin resistance or animal models (see Annex 4). Again, ex vivo/in vivo testing involving patients allergic to the source material may also be considered.

The FAO/WHO 2001 decision tree is not applicable to the evaluation of foods where gene products are down-regulated for hypoallergenic purposes. In such cases, in vivo testing including skin prick testing, open challenges, and double-blind, placebo-controlled food challenges would be required.

5.4. Food containing a gene derived from a source not known to be allergenic

When the expressed protein comes from a source that is not known to be allergenic, the FAO/WHO 2001 decision tree focuses on (1) sequence homology to known allergens (food and environmental), (2) targeted serum screening for cross-reactivity with sera from patients allergic to materials that are broadly related to the source material for the gene, (3) pepsin resistance and (4) immunogenicity testing in animal models (Annex 4). In this situation the search for homologous allergens is based on two procedures.

The first step is a database search for an allergen with a homologous amino acid sequence, according to the principles described in Section 6.1. If this search reveals a level of homology with a known allergen that suggests a potential for cross-reactivity, the expressed protein is considered to be an allergenic risk. No further evaluation for allergenicity would typically be necessary.

The second step is conducted if no such homologous protein is found. In such cases, cross-reactivity is tested with a panel of serum samples that contain high levels of IgE antibodies with a specificity that is broadly related to the gene source (Section 6.3). For this "targeted serum screen", 6 groups of source organisms are distinguished: yeast/moulds, monocots, dicots, invertebrates, vertebrates and "others". A panel of 50 serum samples with high levels of IgE to allergens in the relevant group is used to search for IgE antibodies that are cross-reactive with the expressed protein. If a positive reaction is obtained with one of these sera, the expressed protein is considered to be an allergenic risk and further evaluation for allergenicity would typically not be necessary. If a gene were obtained from a bacterial source, no targeted serum screening would be possible, since no normal population of individuals are known to be sensitised (IgE mediated) to bacterial proteins.

When a positive outcome is obtained in targeted serum screening, further evaluation using in vivo/ex vivo approaches as described in Section 5.3 may be conducted if desired to seek confirmation of the results of the targeted serum screening. If the results obtained with in vivo/ex vivo testing differ from those obtained with targeted serum screening, these results would be more convincing than a positive outcome in the targeted serum screening provided that appropriate, well documented allergic subjects were used in the in vivo/ex vivo testing.

If no cross-reactive serum is found, the protein is analysed for pepsin resistance and for evidence of immunogenicity in appropriate animal models according to the protocols provided in Sections 6.4 and 6.5.

5.5. Post marketing surveillance

The Consultation acknowledges that the pre-market allergenicity assessment of the genetically modified food gives a satisfactory safety assurance. However, it is recognised that due to the wide genetic variability in the human population and different geographical dietary intake, further evaluation for adverse effects of the genetically modified food should be considered once the product has reached the market. This could provide additional safety assurance.

Ideally, a notifying, self-reporting system for any adverse health effects, both for consumers and for employees in the food production industry should be put in place. Reported data should be validated with respect to:

- the clinical outcome in relation to allergenicity
- the causality between the reported adverse effect and the specific genetically modified food/food ingredient exposure

These validated data should be recorded, consolidated and published. Such a system could benefit from experiences of existing national surveillance systems (e.g. disease control centres, poisoning centres).

However, the feasibility of post-marketing surveillance systems should be further explored, since there are number of problems to be addressed, including:

- traceability and labelling of the genetically modified food/food ingredient
- lack of background data on prevalence and incidence in food related allergies
- existence of many confounding food and non-food related factors
- changes in diets over time
- lack of trained experts and infrastructure, especially in developing country settings

5.6. Other Criteria that were Considered

5.6.1. Level of expression

Highly allergenic proteins are often expressed at relatively high levels. However, allergens can sensitize susceptible individuals at less than milligram levels, possibly at less than microgram levels (Sorva et al., 1994; Jarvinen et al., 1999). The elicitation of objective symptoms in already sensitized individuals can also occur at low levels of exposure, but has not been documented below 500 micrograms (Rance and Dutau, 1997; Hourihane et al., 1997). It is therefore not possible to define a level of expression below which a protein can be considered safe from the allergenicity point of view. Thus, level of expression cannot yet be incorporated into the assessment of the allergenicity of genetically modified foods.

5.6.2. Unintended effects

In achieving the objective of conferring a specific target trait (intended effect) to the host organism by the insertion of DNA sequences, additional traits could, theoretically, be acquired or existing traits lost or enhanced (unintended effects). Unintended effects may be due to factors such as random insertion events, which might result in disruption of existing genes and

modification of protein expression. While unintended effects are not specific to the use of recombinant DNA techniques, any such effects should be identified to the maximum extent possible and their impact upon the allergenicity of the genetically modified food should be assessed.

With respect to allergenicity, two types of unintended effects could be envisioned. First, the gene insert may activate or suppress existing host genes in an inordinate fashion leading to either over-expression or under-expression of specific proteins. If the host plant contains known allergenic proteins, then the possibility that the levels of these allergens has been elevated should be considered as part of the safety evaluation process. Secondly, if evidence is obtained from comparison of the genetically modified food to its conventional counterpart that the insertion of the gene creates additional new proteins, then these proteins should be evaluated for their potential allergenicity using the approach described herein.

6. Standardization of Methodologies

6.1. Sequence Homology as Derived from Allergen Databases

The commonly used protein databases (PIR, SwissProt and TrEMBL) contain the amino acid sequences of most allergens for which this information is known. However, these databases are currently not fully up-to-date. A specialized allergen database is under construction.

Suggested procedure on how to determine the percent amino acid identity between the expressed protein and known allergens.

Step 1: *obtain the amino acids sequences of all allergens in the protein databases (for SwissProt and TrEMBL: see http://expasy.ch/tools; for PIR see http://www-nbrf.georgetown.edu/pirwww) in FASTA-format (using the amino acids from the mature proteins only, disregarding the leader sequences, if any). Let this be data set (1).*

Step 2: *prepare a complete set of 80-amino acid length sequences derived from the expressed protein (again disregarding the leader sequence, if any). Let this be data set (2).*

Step 3: *go to EMBL internet address: http://www2.ebi.ac.uk and compare each of the sequences of the data set (2) with all sequences of data set (1), using the FASTA program on the web site for alignment with the default settings for gap penalty and width.*

Cross-reactivity between the expressed protein and a known allergen (as can be found in the protein databases) has to be considered when there is:

1) more than 35 % identity in the amino acid sequence of the expressed protein (i.e. without the leader sequence, if any), using a window of 80 amino acids and a suitable gap penalty (using Clustal-type alignment programs or equivalent alignment programs)

or:

2) identity of 6 contiguous amino acids.

If any of the identity scores equals or exceeds 35 %, this is considered to indicate significant homology within the context of this assessment approach. The use of amino acid

sequence homologies to identify prospective cross-reacting allergens in genetically modified foods has been discussed in more detail elsewhere (Gendel, 1998a; Gendel, 1998b).

Structural similarity with known allergens may still be important if significant amino acid identity is found, but it is below 35 %. In this case significant cross-reactivity is unlikely. However, some families of structurally related proteins are known to contain several allergens. Some examples are:

- lipocalins
- non-specific lipid transfer proteins
- napins (2S albumins from seeds)
- parvalbumins.

If the expressed protein belongs to such a family, it may be considered to have a higher probability to be an allergenic protein.

Functional similarity without structural similarity is unlikely to result in significant cross-reactivity. For example, protease inhibitors that belong to distinct protein families are not known to be cross-reactive. Similarly, proteins belonging to structurally unrelated classes of pathogenesis-related proteins (PR-proteins) are not known to be cross-reactive.

Since identity of 6 contiguous amino acids has an appreciable risk of occurring by chance, verification of potential crossreactivity is warranted when criterion (1) is negative, but criterion (2) is positive. In this situation suitable antibodies (from human or animal source) have to be tested to substantiate the potential for crossreactivity.

6.2. Specific serum screening

In the evaluation of the reactivity of IgE antibodies in the sera of patients with known allergies to relevant source materials, an appropriate in vitro method should be applied. A variety of well validated immunoassays are available for this purpose. The Consultation agrees that any of these tests can be used.

In addition to the precautions cited earlier with respect to selection of suitable sera for such screening, the importance of glycosylation and glycan epitopes must also be considered. Proteins to be expressed in plant hosts may be posttranslationally modified, which may have an impact on their allergenic potential. The effects of glycosylation are particularly relevant to consider, because:

1. The degree of glycosylation may affect the susceptibility of the protein to processing and proteolysis;
2. Glycosylation may alter the epitope structure, either by shielding part of the protein surface (particularly if the glycosylation is extensive), or by introducing glycan epitopes. Glycan epitopes are known to be highly cross-reactive

Glycans may be attached either via an N-link or via an O-link. N-linked sites can be predicted with some accuracy, but the prediction of sites for O-glycosylation is still unreliable.

Cross-reactivity of IgE antibodies to glycan epitopes is important not so much because of their potential contribution to allergic symptomatology (which may be minimal in many cases), but because the structure of the protein part of these glycoproteins is in this situation largely irrelevant: all proteins with these glycan structures will be cross-reactive. When target glycoproteins are screened for cross-reactivity, it is important to make a clear distinction between IgE antibodies to the glycan part on the one hand and IgE antibodies to the protein part

on the other hand. In general, it is advisable to select serum samples without IgE antibodies to glycans, absorb out such IgE antibodies with irrelevant glycoproteins obtained from the same host, or perform such tests with non-glycosylated variants, e.g. expressed in a bacterial host.

Information on glycan epitopes in relation to allergy is largely based on work with plant glycoproteins and invertebrate glycoproteins. Less is known about glycoproteins of eukaryotic microorganisms such as yeast. However, it is likely that similar precautions may need to be taken.

6.3. Targeted serum screening

When no sequence homology has been found between the expressed protein and an allergen, this does not mean that there is no such homologous allergen. It may be due to a lack of information on the relevant allergen. Random screening of serum samples from the allergic population is unlikely to be rewarding. However, some more targeted approach may, in some situations, be more appropriate.

- If the recombinant protein is derived from a monocot, it is proposed to test serum samples from patients with high levels of IgE antibodies to monocot allergens such as grass and rice.
- If the recombinant protein is derived from a dicot, it is proposed to test serum samples from patients with high levels of IgE antibodies to dicot allergens such as tree pollen, weed pollen, celery, peanuts, tree nuts and latex.
- If the allergen is derived from a mould, it is proposed to test serum samples from patients with high levels of IgE antibodies to moulds, yeast and fungi, such as Alternaria or Cladosporium, and of patients with aspergillosis or Trichophyton sensitivity.
- If the allergen is derived from an invertebrate, it is proposed to test serum samples from patients with high levels of IgE antibodies to invertebrates such as mites, cockroach, shrimp, chironimids or silk.
- If the allergen is derived from a vertebrate, it is proposed to test serum samples from patients with high levels of IgE antibodies to mammalian pets, laboratory animals, cow's milk, fish, chicken egg white and chicken egg yolk/serum proteins.
- If the allergen is derived from another source, e.g. a bacterium, no general screen using targeted sera is currently available.

The use of large serum pools (> 5 sera) is discouraged, because this will dilute any cross-reactive antibody present. For maximal sensitivity, individual sera should be tested.

Typically, a screen with 25 individual serum samples with high levels of IgE to the selected group of airborne allergens and (if applicable) 25 with IgE to the selected group of food allergens would be used.

6.4. Pepsin Resistance

Purified or enriched expressed protein (non-heated and non-processed) should be subjected to pepsin degradation conditions using Standard Operating Procedures and Good Laboratory Practices (SOP/GLP). In addition, the expressed protein should be assessed in its principal edible form under identical pepsin degradation conditions to those used to examine the expressed protein. Both known non-allergenic (soybean lipoxygenase, potato acid phosphatase or equivalent) and allergenic (milk beta lactoglobulin, soybean trypsin inhibitor or equivalent) food proteins should be included as comparators to determine the relative degree of the expressed proteins pepsin resistance. The protein concentrations should be assessed using a colorimetric

assay (e.g., Bicinchoninic acid assay (BCA), Bradford Protein Assay, or equivalent protein assay) with bovine serum albumin (BSA) as a standard. Pepsin proteolytic activity should be assessed (Ryle). Enzyme/protein mixtures should be prepared using 500 µg of protein in 200 µL of 0.32% pepsin (w/v) in 30 mM/L NaCl, pH 2.0, and maintained in a shaking 37 C water bath for 60 minutes. Individual 500 microgram aliquots of pepsin/protein solution should be exposed for periods of 0, 15, 30 seconds and 1, 2, 4, 8, 15, and 60 minutes, at which time each aliquot should be neutralised with an appropriate buffer. Neutralised protein solutions should be mixed with SDS-PAGE sample loading buffer with and without reducing agent (DTT or 2-ME) and heated for 5 minutes at 90°C. Samples containing 5µg/cm gel of protein should be evaluated using 10-20% gradient Tricine SDS-PAGE gels or equivalent gel system under both non-reducing and reducing electrophoretic conditions. Protein in the gels should be visualised by silver or colloidal gold staining procedures. Evidence of intact expressed protein and/or intact fragments greater than 3.5 kDa would suggest a potential allergenic protein. Evidence of protein fragments less than 3.5 kDa would not necessarily raise issues of protein allergenicity and the data should be taken into consideration with other decision tree criteria. For detection of expressed protein in an edible food source, a polyclonal IgG immunoblot analysis should be performed according to the laboratory procedures. The immunoblot analysis should be compared to the silver or colloidal gold stained SDS-PAGE gel and reflect the stained pattern of the expressed protein run under identical conditions.

The investigator should be aware of and consider the following precautions. Edible food sources may contain protease inhibitors or other substances that may promote or reduce protein degradation. Resulting fragments may not be reactive with the polyclonal IgG antibody source. Finally, there is no absolute certainty that pepsin resistance or complete degradation of a protein will predict the allergenicity of novel proteins and must be taken into consideration with other decision tree criteria. Although the present pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used for which adequate justification is provided. The producer is expected to take these results into consideration in combination with other decision tree criteria.

6.5. Animal Models

For additional assessment of the potential allergenicity of expressed proteins, informative data can be generated using animal models in development. A number of animal models may be considered to assess on a relative scale the potential allergenicity using oral sensitisation routes with the Brown Norway rat model (Knippels et al., 1998) or intraperitoneal administration in murine models (Dearman et al 2000) or other relevant animal models. Results should be presented in characteristic Th1/Th2 antibody (isotype) profiles for assessing the potential immunogenic/allergenic activity. The different routes of administration in animal models (oral versus intraperitoneal) may not give the same results. Therefore, selection of one route of administration is not meant to exclude other routes of sensitisation. It is recommended to consider the results from two sensitisation routes in the same or different animal species.

It is recommended that the potential allergenicity of the expressed protein be ranked against well known strong and weak food allergens and non-allergenic proteins in the animal model. As additional information becomes available with respect to animal models, protocols may need to be modified to give optimal conditions for assessing protein allergenicity.

Although the present animal models provide additional information on potential allergenicity of novel proteins, they do not reflect all aspects of IgE-mediated food allergies in humans.

7. Conclusions

1. The Consultation agreed that the safety assessment of foods derived from biotechnology requires an integrated and stepwise, case-by-case approach, and that this method also be applied to the evaluation of the allergenicity of food derived from biotechnology.
2. The Consultation emphasized that all foods derived from biotechnology must be assessed for allergenic potential.
3. The original decision tree from the FAO/WHO 2000 Consultation served as a basis for this consultation. The Consultation concurred that this decision tree be modified as a consequence of more recent research and which is reflected in the FAO/WHO 2001 decision tree.
4. When the expressed protein is derived from a source with known allergenicity, the FAO/WHO 2001 decision tree proposes that the initial investigation be analysis of sequence homology to known allergens in the source. If this is negative, the next step will be investigations on possible IgE binding using immunoassays and may also include investigations in vivo in patients allergic to the source food.
5. When the expressed protein is derived from a source with no known allergenicity, the FAO/WHO 2001 decision tree proposes that the initial investigation would also be analysis of sequence homology to known allergens from food and environmental sources. If positive matches are found with known allergens, then the protein is considered likely allergenic. If no significant sequence homology is identified, then targeted serum screening is conducted with serum samples that contain high levels of IgE antibodies with a specificity that is broadly related to the gene source. If the targeted serum screening is positive, then the protein is considered likely allergenic. If the targeted serum screening is negative, then pepsin resistance of the expressed protein and the immunogenicity of the expressed protein in suitable animal models are to be assessed to determine the likelihood that the protein will be allergenic.
6. The Consultation agreed that the FAO/WHO 2001 decision tree is not applicable to the evaluation of foods where hypo-allergenicity has been induced by down-regulation of genes.
7. The Consultation was of the opinion that an evaluation of proteins for sequence homology with sufficient sensitivity and specificity to detect potential cross-reactivity is an important part of the process for the assessment of the allergenicity of the expressed protein.
8. The Consultation agreed that further studies would be required to determine the amount of allergen that sensitises and elicits allergic events.
9. The Consultation recognized the need to constantly update allergen databases.
10. The Consultation concluded that animal models have not been evaluated for all food allergens but there is sufficient scientific evidence that using these models will contribute valuable information regarding the allergenicity of foods derived from biotechnology.
11. The Consultation agreed that pepsin susceptibility is a relevant parameter for the identification of potential allergens and that the protocol described is not intended to mimic the physiologic conditions of gastric digestion.

12. The use of human in vivo methods to evaluate the allergenicity of foods derived from biotechnology may in many circumstances raise ethical issues and their use will have to be considered on a case-by-case basis.

13. Post-market surveillance is a valuable tool in the monitoring of adverse effects and long-term sequelae of foods derived from biotechnology and the Consultation recognized that the feasibility of certain aspects of its implementation would need further investigation.

14. The Consultation accepted that the FAO/WHO 2001 decision tree and its accompanying clarifying text will require modification in the future as a result of the rapidly expanding scientific base in the allergy and biotechnology fields but that this decision tree is appropriate based on our present knowledge.

8. Recommendations

1. The Consultation recommends that the FAO/WHO 2001 decision tree be used for determining allergenicity of foods derived from biotechnology.
2. The Consultation recommends that FAO and WHO should endeavour to update the decision tree as and when required.
3. The identification of food allergens and the characteristics of these allergens that define their immunogenicity are encouraged.
4. Protein and gene databases required for the assessment of allergenicity of foods derived from biotechnology should be frequently updated and maintained.
5. Further research is needed on the development and validation of suitable animal models and procedures for the assessment of allergenicity of foods derived from biotechnology.
6. The Consultation recommends that the possibility of implementing post-marketing surveillance should be further studied.
7. The Consultation recommends that FAO and WHO provide technical support to member countries to strengthen their capacity and infrastructure to enable those countries to undertake the evaluation of the allergenicity of foods derived from biotechnology.
8. The Consultation recommends to FAO and WHO the establishment of a coordination network to promote and strengthen the interaction between experts to improve standard operating procedures, good laboratory practices and good clinical practice to facilitate the evaluation of the allergenicity of foods derived from biotechnology.

9. List of Abbreviations

BCA: Bicinchoninic acid assay

BSA: Bovine Serum Albumin

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EAACI: European Academy of Allergology and Clinical Immunology

FAO: Food and Agriculture Organization of the United Nations

GM: Genetically Modified

GLP: Good Laboratory Practices

IFBC: International Food Biotechnology Council

Ig: Immunoglobulin

IgE: Immunoglobulin E

IgG: Immunoglobulin G

ILSI: International Life Science Institute

kIU/L: Kilointernational Units/Litre

kDa: Kilodalton

ME: Mercaptoethanole

OAS: Oral allergy syndrome

PR-proteins: Pathogenesis-Related proteins

Th1: T-helper lymphocytes 1, which assist the differentiation of cytotoxic cells and also activate macrophages, which after activation play a role as effectors of the immune response.

Th2: T-helper lymphocytes 2, which are mainly involved in the amplification of B lymphocyte responses.

SCOOP/NUTR/REPORT/2: Scientific Cooperation Programme/Nutrition/Report/2

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SOP: Standard Operating Procedures

WHO: World Health Organization

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Annex 1

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Annex 2

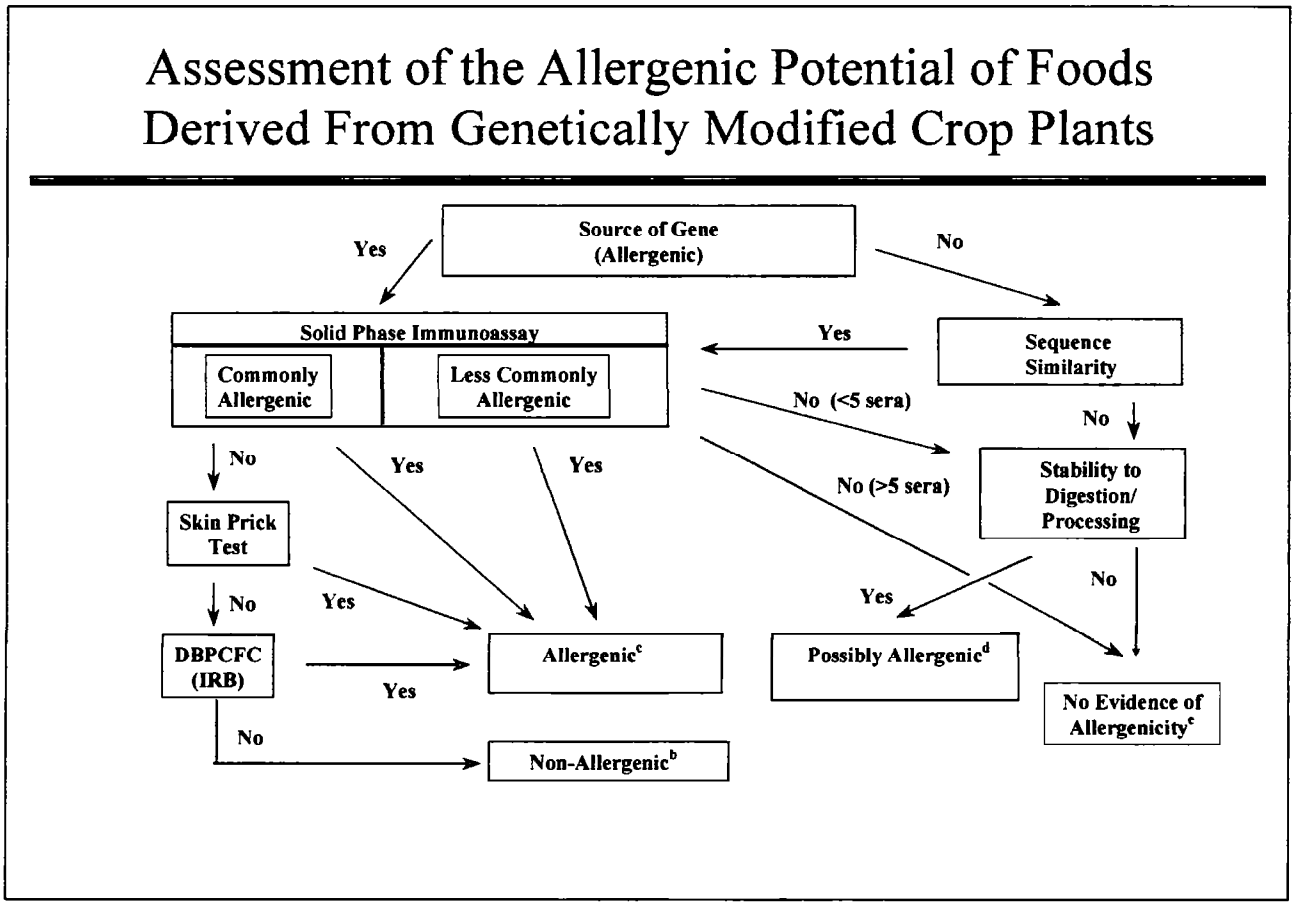
List of Documents[13]

Biotech 01/01	Provisional Agenda and Timetable
Biotech 01/02	Questions about the Assessment of Allergenicity of Foods Derived from Modern Biotechnology
Biotech 01/03	Topic 1: Overview of the Current Approach to Determine the Allergenicity of Genetically Modified Foods (Decision Tree Approach)
Biotech 01/04	Topic 2: Commonly Known Allergenic Sources (IgE-Mediated and Non IgE-Mediated Food Allergens as well as Environmental Allergens)
Biotech 01/05	Topic 3: Allergen Databases/Class of Proteins/Allergen Function
Biotech 01/06	Topic 4: Sequence Homology and Allergen Structure
Biotech 01/07	Topic 5: Stability of Known Allergens (Digestion and Heat Stability
Biotech 01/08	Topic 6: Solid phase Immunoassay, Immunoreactivity and Other Criteria
Biotech 01/09	Topic 7: Prevalence of Allergen in Food and Threshold for Sensitization
Biotech 01/10	Topic 8: Animal Model for Allergenicity Assessment
Biotech 01/11	Topic 9: Post-market Surveillance of Allergenicity

[13] Working Documents are posted on the following FAO and WHO websites:
FAO : http://www.fao.org/WAICENT/FAOINFO/ECONOMIC/ESN/biotech.htm
WHO: http://www.who.int/fsf

Annex 3

FAO/WHO 2000 Decision Tree



Footnotes to Figure

(a) The figure was adapted from decision-tree approach developed by International Food Biotechnology Council and Allergy and Immunology Institute of the International Life Sciences Institute (Metcalfe *et al.*, 1996).

(b) The combination of tests involving allergic human subjects or blood serum from such subjects would provide a high level of confidence that no major allergens were transferred. The only remaining uncertainty would be the likelihood of a minor allergen affecting a small percentage of the population allergenic to the source material.

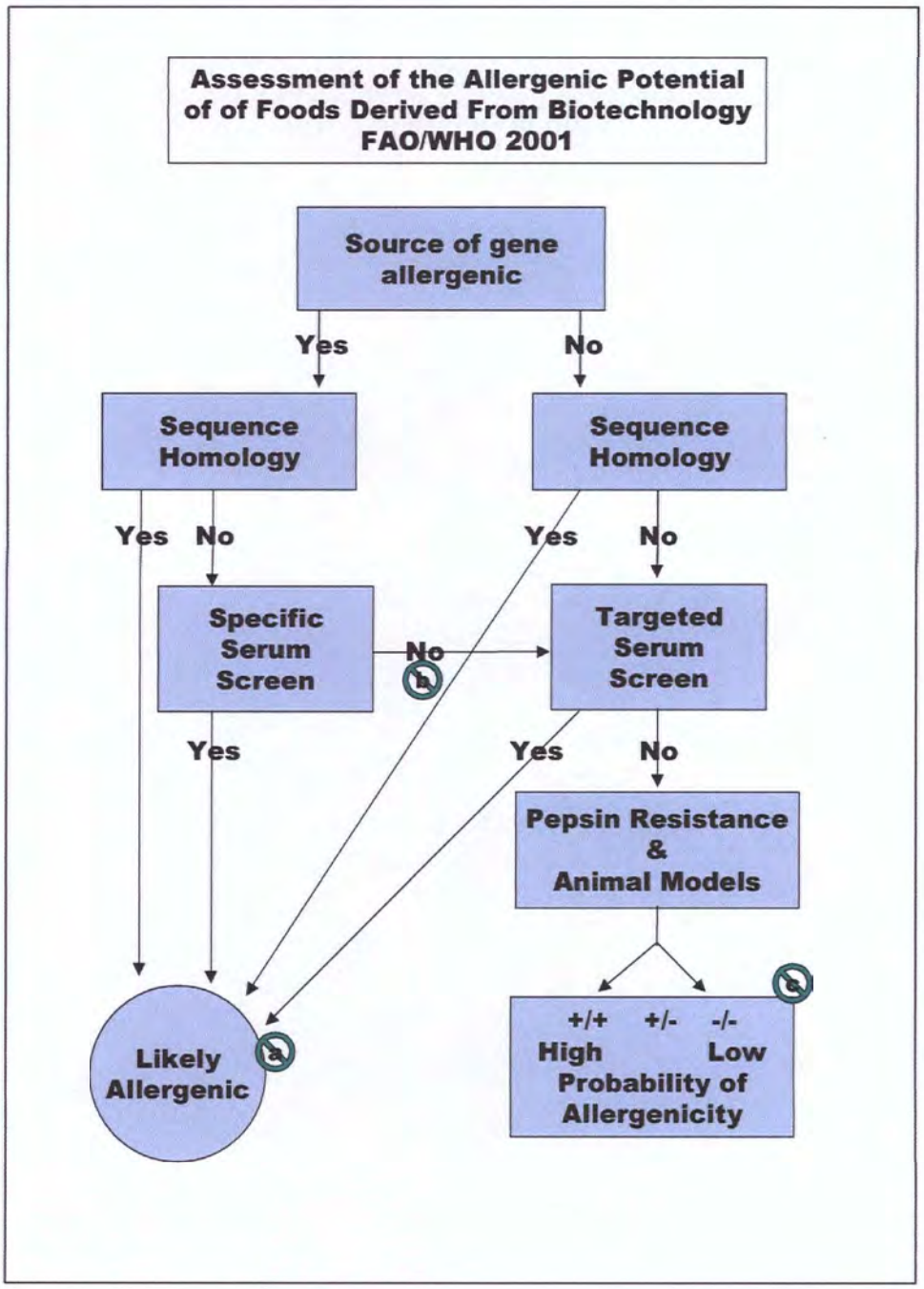
(c) Any positive results obtained in tests involving allergenic human subjects or blood serum from such subjects would provide a high level of confidence that the novel protein was a potential allergen. Foods containing such novel proteins would need to be labelled to protect allergic consumers.

(d) A novel protein with either no sequence similarity to known allergens or derived from a less commonly allergenic source with no evidence of binding to IgE from the blood serum of a few allergic individuals (<5), but that is stable to digestion and processing should be considered a possible allergen. Further evaluation would be necessary to address this uncertainty. The nature of the tests would be determined on a case-by-case basis.

(e) A novel protein with no sequence similarity to known allergens and that was not stable to digestion and processing would have no evidence of allergenicity. Similarly, a novel protein expressed by a gene obtained from a less commonly allergenic source and demonstrated to have no binding with IgE from the blood serum of a small number of allergic individuals (>5 but <14) provides no evidence of allergenicity. Stability testing may be included in these cases. However, the level of confidence based on only two decision criteria is modest. The Consultation suggested that other criteria should also be considered such as the level of expression of the novel protein.

Annex 4

FAO/WHO 2001 Decision Tree



Footnotes

[a] Any positive results obtained from sequence homology comparisons to the sequences of known allergens in existing allergen databases or from serum screening protocols, both conducted in accordance with the guidelines established in Sections 6.1, 6.2 and 6.3, indicate that the expressed protein is likely allergenic.

[b] The degree of confidence in negative results obtained in the specific serum screen is enhanced by the examination of larger numbers of individual sera as explained in Section 5.3. Conducting the specific serum screen with small numbers of individual sera when larger numbers of such sera are readily available should be discouraged.

[c] When positive results are obtained in both the pepsin resistance and animal model protocols, the expressed protein has a high probability to become an allergen. When negative results are obtained in both protocols, the expressed protein is unlikely to become an allergen. When different results are obtained in the pepsin resistance and animal model protocols, the probability of allergenicity is intermediate, although rational explanations may be possible in some situations.

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Second edition

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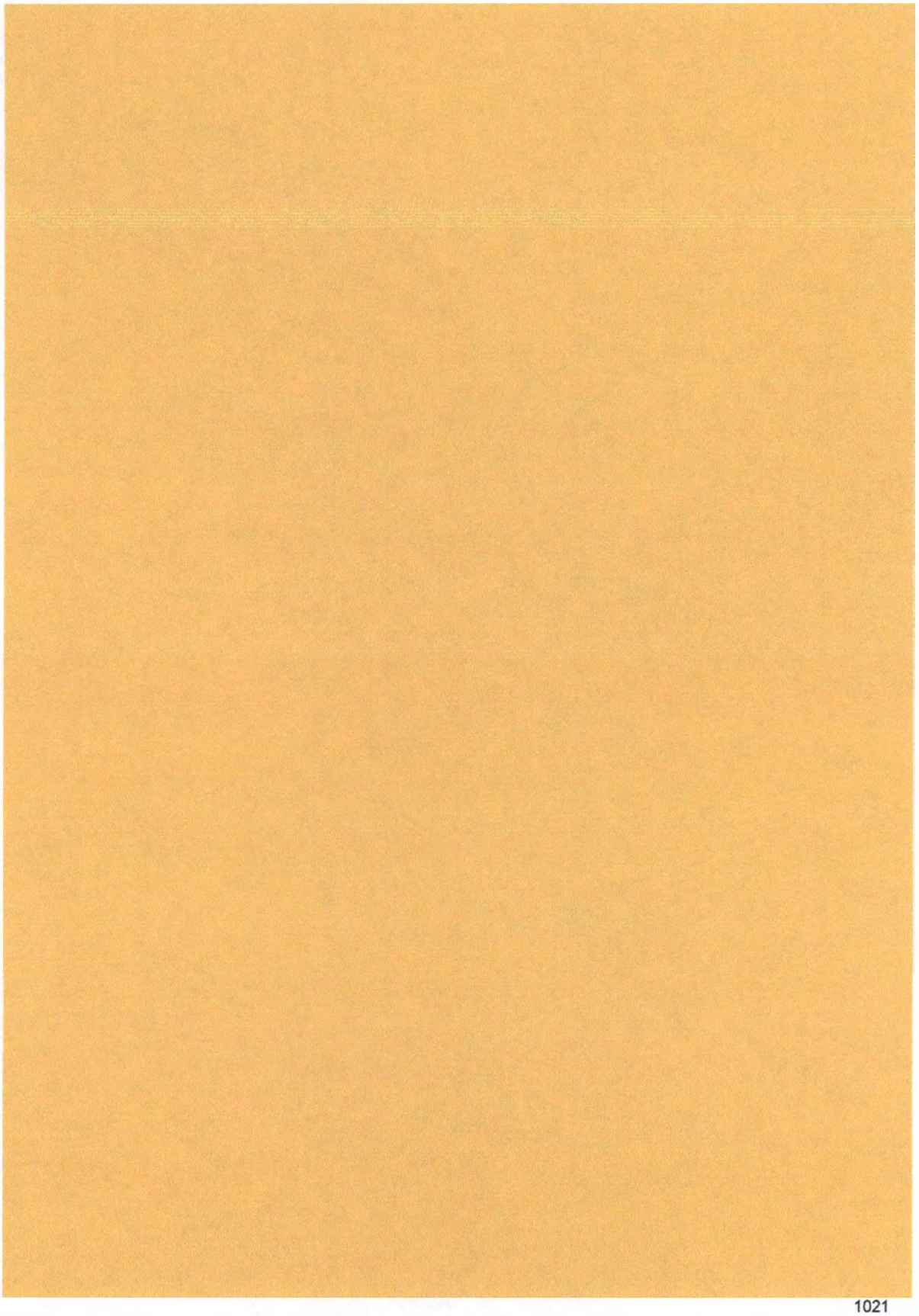

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CODEX ALIMENTARIUS

Foods derived from modern biotechnology

Second edition

WORLD HEALTH ORGANIZATION

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

Rome, 2009

1022

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THE CODEX ALIMENTARIUS COMMISSION

The Codex Alimentarius Commission is an intergovernmental body with more than 180 members, within the framework of the Joint Food Standards Programme established by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), with the purpose of protecting the health of consumers and ensuring fair practices in the food trade. The Commission also promotes coordination of all food standards work undertaken by international governmental and non-governmental organizations.

The *Codex Alimentarius* (Latin, meaning Food Law or Code) is the result of the Commission's work: a collection of internationally adopted food standards, guidelines, codes of practice and other recommendations. The texts in this publication are part of the Codex Alimentarius.

FOODS DERIVED FROM MODERN BIOTECHNOLOGY
Second edition

The texts in this publication represent the outcome of the work of the Codex Alimentarius Commission on principles and guidelines for food safety assessment of foods derived from modern biotechnology. They give guidance on how to assess the safety of such foods and thus protect the health of consumers. This second edition includes texts adopted by the Codex Alimentarius Commission up to 2008.

Further information on these texts, or any other aspect of the Codex Alimentarius Commission, may be obtained from:

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CONTENTS

FOODS DERIVED FROM MODERN BIOTECHNOLOGY
Second edition

PRINCIPLES FOR THE RISK ANALYSIS OF FOODS DERIVED FROM MODERN BIOTECHNOLOGY

CAC/GL 44-2003

SECTION 1 – INTRODUCTION

1. For many foods, the level of food safety generally accepted by society reflects the history of their safe consumption by humans. It is recognized that in many cases the knowledge required to manage the risks associated with foods has been acquired in the course of their long history of use. Foods are generally considered safe provided that care is taken during development, primary production, processing, storage, handling and preparation.

2. The hazards associated with foods are subjected to the risk analysis process of the Codex Alimentarius Commission to assess potential risks and, if necessary, to develop approaches to manage these risks. The conduct of risk analysis is guided by general decisions of the Codex Alimentarius Commission[1] as well as the *Working Principles for risk analysis*.[2]

3. While risk analysis has been used over a long period of time to address chemical hazards (e.g. residues of pesticides, contaminants, food additives and processing aids), and it is being increasingly used to address microbiological hazards and nutritional factors, the principles were not elaborated specifically for whole foods.

4. The risk analysis approach can, in general terms, be applied to foods including foods derived from modern biotechnology. However, it is recognized that this approach must be modified when applied to a whole food rather than to a discrete hazard that may be present in food.

5. The principles presented in this document should be read in conjunction with the *Working Principles for risk analysis* to which these principles are supplemental.

6. Where appropriate, the results of a risk assessment undertaken by other regulatory authorities may be used to assist in the risk analysis and avoid duplication of work.

[1] These decisions include the "Statements of principle concerning the role of science in the Codex decision-making process and the extent to which other factors are taken into account" and the "Statements of principle relating to the role of food safety risk assessment" (*Codex Alimentarius Commission Procedural Manual*, 13th edition).

[2] *Working principles for risk analysis for application in the framework of the Codex Alimentarius* (adopted by the 26th Session of the Codex Alimentarius Commission, 2003; *Codex Alimentarius Commission Procedural Manual*, 13th edition).

Adopted 2003. Amendment 2008.

SECTION 2 – SCOPE AND DEFINITIONS

7. The purpose of these Principles is to provide a framework for undertaking risk analysis on the safety and nutritional aspects of foods derived from modern biotechnology. This document does not address environmental, ethical, moral and socio-economic aspects of the research, development, production and marketing of these foods.[3]

8. The definitions below apply to these Principles:

Modern biotechnology means the application of:

i) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles; or

ii) fusion of cells beyond the taxonomic family that overcome natural physiological reproductive or recombinant barriers and that are not techniques used in traditional breeding and selection.[4]

Conventional counterpart means a related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food.[5]

SECTION 3 – PRINCIPLES

9. The risk analysis process for foods derived from modern biotechnology should be consistent with the *Working Principles for risk analysis*.

Risk assessment

10. Risk assessment includes a safety assessment, which is designed to identify whether a hazard, nutritional or other safety concern is present, and if present, to gather information on its nature and severity. The safety assessment should include a comparison between the food derived from modern biotechnology and its conventional counterpart, focusing on determination of similarities and differences. If a new or altered hazard, nutritional or other safety concern is identified by the safety assessment, the risk associated with it should be characterized to determine its relevance to human health.

11. A safety assessment is characterized by an assessment of a whole food or a component thereof relative to the appropriate conventional counterpart:

A. taking into account both intended and unintended effects;
B. identifying new or altered hazards;
C. identifying changes relevant to human health in key nutrients.

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[3] This document does not address animal feed and animals fed such feed except insofar as these animals have been developed by using modern biotechnology.

[4] This definition is taken from the *Cartagena Biosafety Protocol* under the Convention on Biological Diversity.

[5] It is recognized that, for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

12. A pre-market safety assessment should be undertaken following a structured and integrated approach and be performed on a case-by-case basis. The data and information, based on sound science, obtained using appropriate methods and analysed using appropriate statistical techniques, should be of a quality and, as appropriate, of a quantity that would withstand scientific peer review.

13. Risk assessment should apply to all relevant aspects of foods derived from modern biotechnology. The risk assessment approach for these foods is based on a consideration of science-based multidisciplinary data and information taking into account the factors mentioned in the accompanying Guidelines.[6]

14. Scientific data for risk assessment are generally obtained from a variety of sources, such as the developer of the product, scientific literature, general technical information, independent scientists, regulatory agencies, international bodies and other interested parties. Data should be assessed using appropriate science-based risk assessment methods.

15. Risk assessment should take into account all available scientific data and information derived from different testing procedures, provided that the procedures are scientifically sound and the parameters being measured are comparable.

Risk management

16. Risk management measures for foods derived from modern biotechnology should be proportional to the risk, based on the outcome of the risk assessment and, where relevant, taking into account other legitimate factors in accordance with the general decisions of the Codex Alimentarius Commission[7] as well as the *Working Principles for risk analysis*.

17. It should be recognized that different risk management measures may be capable of achieving the same level of protection with regard to the management of risks associated with safety and nutritional impacts on human health, and therefore would be equivalent.

18. Risk managers should take into account the uncertainties identified in the risk assessment and implement appropriate measures to manage these uncertainties.

19. Risk management measures may include, as appropriate, food labelling[8] conditions for marketing approvals and post-market monitoring.

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[6] Reference is made to the *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants* (CAC/GL 45-2003), the *Guideline for the conduct of food safety assessment of foods produced using recombinant-DNA micro-organisms* (CAC/GL 46-2003) and the *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA animals* (CAC/GL 68-2008).

[7] See footnote 1.

[8] Reference is made to the Codex Committee on Food Labelling (CCFL) in relation to the proposed *Draft Guidelines for the labelling of foods and food ingredients obtained through certain techniques of genetic modification/genetic engineering* at Step 3 of the Codex Elaboration Procedure.

20. Post-market monitoring may be an appropriate risk management measure in specific circumstances. Its need and utility should be considered, on a case-by-case basis, during risk assessment and its practicability should be considered during risk management. Post-market monitoring may be undertaken for the purpose of:
   A. verifying conclusions about the absence or the possible occurrence, impact and significance of potential consumer health effects; and
   B. monitoring changes in nutrient intake levels, associated with the introduction of foods likely to alter nutritional status significantly, to determine their human health impact.

21. Specific tools may be needed to facilitate the implementation and enforcement of risk management measures. These may include appropriate analytical methods; reference materials; and, the tracing of products[9] for the purpose of facilitating withdrawal from the market when a risk to human health has been identified or to support post-market monitoring in circumstances as indicated in paragraph 20.

Risk communication

22. Effective risk communication is essential in all phases of risk assessment and risk management. It is an interactive process involving all interested parties, including government, industry, academia, media and consumers.

23. Risk communication should include transparent safety assessment and risk management decision-making processes. These processes should be fully documented at all stages and open to public scrutiny, whilst respecting legitimate concerns to safeguard the confidentiality of commercial and industrial information. In particular, reports prepared on the safety assessments and other aspects of the decision-making process should be made available to all interested parties.

24. Effective risk communication should include responsive consultation processes. Consultation processes should be interactive. The views of all interested parties should be sought and relevant food safety and nutritional issues that are raised during consultation should be addressed during the risk analysis process.

Consistency

25. A consistent approach should be adopted to characterize and manage safety and nutritional risks associated with foods derived from modern biotechnology. Unjustified differences in the level of risks presented to consumers between these foods and similar conventional foods should be avoided.

[9] It is recognized that there are other applications of product tracing. These applications should be consistent with the provisions of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) and the Agreement on Technical Barriers to Trade (TBT Agreement). The application of product tracing to the areas covered by both Agreements was considered by the Codex Committee on Food Import and Export Inspection and Certification Systems, see: *Principles for traceability / product tracing as a tool within a food inspection and certification system* (CAC/GL 60-2006).

26. A transparent and well-defined regulatory framework should be provided in characterizing and managing the risks associated with foods derived from modern biotechnology. This should include consistency of data requirements, assessment frameworks, the acceptable level of risk, communication and consultation mechanisms and timely decision processes.

Capacity building and information exchange

27. Efforts should be made to improve the capability of regulatory authorities, particularly those of developing countries, to assess, manage and communicate risks, including enforcement, associated with foods derived from modern biotechnology or to interpret assessments undertaken by other authorities or recognized expert bodies, including access to analytical technology. In addition, capacity building for developing countries, either through bilateral arrangements or with assistance of international organizations, should be directed towards effective application of these principles.[10]

28. Regulatory authorities, international organizations and expert bodies and industry should facilitate, through appropriate contact points (including but not limited to Codex Contact Points) and other appropriate means, the exchange of information, including the information on analytical methods.

Review processes

29. Risk analysis methodology and its application should be consistent with new scientific knowledge and other information relevant to risk analysis.

30. Recognizing the rapid pace of development in the field of biotechnology, the approach to safety assessments of foods derived from modern biotechnology should be reviewed when necessary to ensure that emerging scientific information is incorporated into the risk analysis. When new scientific information relevant to a risk assessment becomes available, the assessment should be reviewed to incorporate that information and, if necessary, risk management measures adapted accordingly.

[10] Reference is made to technical assistance of provisions in Article 9 of the SPS Agreement and Article 11 of the TBT Agreement.

GUIDELINE FOR THE CONDUCT OF FOOD SAFETY ASSESSMENT OF FOODS DERIVED FROM RECOMBINANT-DNA PLANTS

CAC/GL 45-2003

SECTION 1 – SCOPE

1. This Guideline supports the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003). It addresses safety and nutritional aspects of foods consisting of, or derived from, plants that have a history of safe use as sources of food, and that have been modified by modern biotechnology to exhibit new or altered expression of traits.

2. This document does not address animal feed or animals fed with the feed. This document also does not address environmental risks.

3. The Codex principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities, such as food additives and pesticide residues, or a specific chemical or microbial contaminant that have identifiable hazards and risks; they are not intended to apply to whole foods as such. Indeed, few foods have been assessed scientifically in a manner that would fully characterize all risks associated with the food. Further, many foods contain substances that would probably be found harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. This approach is based on the principle that the safety of foods derived from new plant varieties, including recombinant deoxyribonucleic acid (DNA) plants, is assessed relative to the conventional counterpart having a history of safe use, taking into account both intended and unintended effects. Rather than trying to identify every hazard associated with a particular food, the intention is to identify new or altered hazards relative to the conventional counterpart.

5. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003). If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and if necessary further risk assessment, the food would be subjected to risk management considerations in accordance with the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003) before it is considered for commercial distribution.

Adopted 2003. Annexes 2 and 3 adopted 2008.

6. Risk management measures such as post-market monitoring of consumer health effects may assist the risk assessment process. These are discussed in paragraph 20 of the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003).

7. The Guideline describes the recommended approach to making safety assessments of foods derived from recombinant-DNA plants where a conventional counterpart exists, and identifies the data and information that are generally applicable to making such assessments. While this Guideline is designed for foods derived from recombinant-DNA plants, the approach described could, in general, be applied to foods derived from plants that have been altered by other techniques.

SECTION 2 – DEFINITIONS

8. The definitions below apply to this Guideline:

Recombinant-DNA plant means a plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

Conventional counterpart means a related plant variety, its components and/or products for which there is experience of establishing safety based on common use as food.[1]

SECTION 3 – INTRODUCTION TO FOOD SAFETY ASSESSMENT

9. Traditionally, new varieties of food plants have not been systematically subjected to extensive chemical, toxicological or nutritional evaluation prior to marketing, with the exception of foods for specific groups, such as infants, where the food may constitute a substantial portion of the diet. Thus, new varieties of corn, soybean, potatoes and other common food plants are evaluated by breeders for agronomic and phenotypic characteristics, but generally, foods derived from such new plant varieties are not subjected to the rigorous and extensive food safety testing procedures, including studies in animals, that are typical of chemicals, such as food additives or pesticide residues, that may be present in food.

10. The use of animal models for assessing toxicological end-points is a major element in the risk assessment of many compounds such as pesticides. However, in most cases, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. Therefore, it is relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels in order to identify any potential adverse health effects of importance to humans. In this way, it is

[1] It is recognized that, for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

11. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, often characterized by a wide variation in composition and nutritional value. Owing to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, this in order to avoid the induction of adverse effects that are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can, therefore, be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

12. Owing to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods derived from food plants, including recombinant-DNA plants. This has been addressed by the development of a multidisciplinary approach for assessing safety that takes into account both intended and unintended changes that may occur in the plant or in the foods derived from it, using the concept of substantial equivalence.

13. The concept of substantial equivalence is a key step in the safety assessment process. However, it is not a safety assessment in itself; rather, it represents the starting point that is used to structure the safety assessment of a new food relative to its conventional counterpart. This concept is used to identify similarities and differences between the new food and its conventional counterpart.[2] It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods derived from recombinant-DNA plants. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the new product can be considered relative to its conventional counterpart.

Unintended effects

14. In achieving the objective of conferring a specific target trait (intended effect) to a plant by the insertion of defined DNA sequences, additional traits could, in some cases, be acquired or existing traits could be lost or modified (unintended effects). The potential occurrence of unintended effects is not restricted to the use of *in vitro* nucleic acid techniques. Rather, it is an inherent and general phenomenon that can

[2] The concept of substantial equivalence as described in the report of the 2000 Joint FAO/WHO Expert Consultation (*Safety aspects of genetically modified foods of plant origin*, WHO/SDE/PHE/FOS/00.6, WHO, Geneva, 2000).

also occur in conventional breeding. Unintended effects may be deleterious, beneficial or neutral with respect to the health of the plant or the safety of foods derived from the plant. Unintended effects in recombinant-DNA plants may also arise through the insertion of DNA sequences and/or they may arise through subsequent conventional breeding of the recombinant-DNA plant. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA plant would have an unexpected adverse effect on human health.

15. Unintended effects can result from the random insertion of DNA sequences into the plant genome, which may cause disruption or silencing of existing genes, activation of silent genes, or modifications in the expression of existing genes. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels may give rise to secondary biochemical effects or changes in the regulation of metabolic pathways and/or altered levels of metabolites.

16. Unintended effects caused by genetic modification may be subdivided into two groups: those that are "predictable" and those that are "unexpected". Many unintended effects are largely predictable based on knowledge of the inserted trait and its metabolic connections or of the site of insertion. Owing to the expanding information on plant genomes and the increased specificity in terms of genetic materials introduced through recombinant-DNA techniques compared with other forms of plant breeding, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse potential changes at the level of gene transcription and message translation that could lead to unintended effects.

17. The safety assessment of foods derived from recombinant-DNA plants involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information is necessary in order to assess unintended effects because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, provide assurance that the food is unlikely to have an adverse effect on human health. The assessment for unintended effects takes into account the agronomic/phenotypic characteristics of the plant that are typically observed by breeders in selecting new varieties for commercialization. These observations by breeders provide a first screen for plants that exhibit unintended traits. New varieties that pass this screen are subjected to safety assessment as described in Sections 4 and 5.

Framework of food safety assessment

18. The safety assessment of a food derived from a recombinant-DNA plant follows a stepwise process of addressing relevant factors that include:

A. description of the recombinant-DNA plant;
B. description of the host plant and its use as food;
C. description of the donor organism(s);

D. description of the genetic modification(s);
E. characterization of the genetic modification(s);
F. safety assessment:
   a) expressed substances (non-nucleic acid substances),
   b) compositional analyses of key components,
   c) evaluation of metabolites,
   d) food processing,
   e) nutritional modification; and
G. other considerations.

19. In certain cases, the characteristics of the product may necessitate development of additional data and information to address issues that are unique to the product under review.

20. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, good laboratory practice. Primary data should be made available to regulatory authorities at request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

21. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. The expected end-point of such an assessment will be a conclusion regarding whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. In essence, therefore, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed and, if so, to make well-informed and appropriate decisions.

SECTION 4 – GENERAL CONSIDERATIONS

Description of the recombinant-DNA plant

22. A description of the recombinant-DNA plant being presented for safety assessment should be provided. This description should identify the crop, the transformation event(s) to be reviewed and the type and purpose of the modification. This description should be sufficient to aid in understanding the nature of the food being submitted for safety assessment.

Description of the host plant and its use as food

23. A comprehensive description of the host plant should be provided. The necessary data and information should include, but need not be restricted to:
A. common or usual name, scientific name and taxonomic classification;
B. history of cultivation and development through breeding, in particular identifying traits that may adversely affect human health;

C. information on the genotype and phenotype of the host plant relevant to its safety, including any known toxicity or allergenicity; and
D. history of safe use for consumption as food.

24. Relevant phenotypic information should be provided not only for the host plant but also for related species and for plants that have made or may make a significant contribution to the genetic background of the host plant.

25. The history of use may include information on how the plant is typically cultivated, transported and stored, whether special processing is required to make the plant safe to eat, and its normal role in the diet (e.g. which part of the plant is used as a food source, whether its consumption is important in particular subgroups of the population, what important macronutrients or micronutrients it contributes to the diet).

Description of the donor organism(s)

26. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health (e.g. presence of antinutrients). The description of the donor organism(s) should include:
A. its usual or common name;
B. scientific name;
C. taxonomic classification;
D. information about the natural history as concerns food safety;
E. information on naturally occurring toxins, antinutrients and allergens; for micro-organisms, additional information on pathogenicity and the relationship to known pathogens; and
F. information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g. possible presence as contaminants).

Description of the genetic modification(s)

27. Sufficient information should be provided on the genetic modification to allow for the identification of all genetic material potentially delivered to the host plant and to provide the necessary information for the analysis of the data supporting the characterization of the DNA inserted in the plant.

28. The description of the transformation process should include:
A. information on the specific method used for the transformation (e.g. *Agrobacterium*-mediated transformation);
B. information, if applicable, on the DNA used to modify the plant (e.g. helper plasmids), including the source (e.g. plant, microbial, viral, synthetic), identity and expected function in the plant; and
C. intermediate host organisms, including the organisms (e.g. bacteria) used to produce or process DNA for transformation of the host organism.

29. Information should be provided on the DNA to be introduced, including:
   A. the characterization of all the genetic components, including marker genes, regulatory and other elements affecting the function of the DNA;
   B. the size and identity;
   C. the location and orientation of the sequence in the final vector/construct; and
   D. the function.

Characterization of the genetic modification(s)

30. In order to provide clear understanding of the impact on the composition and safety of foods derived from recombinant-DNA plants, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out.

31. Information should be provided on the DNA insertions into the plant genome; this should include:
   A. the characterization and description of the inserted genetic materials;
   B. the number of insertion sites;
   C. the organization of the inserted genetic material at each insertion site, including copy number and sequence data of the inserted material and of the surrounding region, sufficient to identify any substances expressed as a consequence of the inserted material, or, where more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food; and
   D. identification of any open reading frames within the inserted DNA or created by the insertions with contiguous plant genomic DNA, including those that could result in fusion proteins.

32. Information should be provided on any expressed substances in the recombinant-DNA plant; this should include:
   A. the gene product(s) (e.g. a protein or an untranslated ribonucleic acid [RNA]);
   B. the function of the gene product(s);
   C. the phenotypic description of the new trait(s);
   D. the level and site of expression in the plant of the expressed gene product(s), and the levels of its metabolites in the plant, particularly in the edible portions; and
   E. where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous messenger RNA (mRNA) or protein.

33. In addition, information should be provided:
   A. to demonstrate whether the arrangement of the genetic material used for insertion has been conserved or whether significant rearrangements have occurred upon integration;
   B. to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;

C. to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable through several generations consistent with laws of inheritance. It may be necessary to examine the inheritance of the DNA insert itself or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly;
D. to demonstrate whether the newly expressed trait(s) are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene;
E. to indicate whether there is any evidence to suggest that one gene (or several genes) in the host plant has been affected by the transformation process; and
F. to confirm the identity and expression pattern of any new fusion proteins.

Safety assessment

Expressed substances (non-nucleic acid substances)

Assessment of possible toxicity

34. *In vitro* nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in plants. The new substances can be conventional components of plant foods, such as proteins, fats, carbohydrates and vitamins, that are novel in the context of that recombinant-DNA plant. New substances might also include new metabolites resulting from the activity of enzymes generated by the expression of the introduced DNA.

35. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations and mean values. Current dietary exposure and possible effects on population subgroups should also be considered.

36. Information should be provided to ensure that genes coding for known toxins or antinutrients present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic or antinutritious characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, as conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate antinutrients or toxicants.

37. For the reasons described in Section 3, conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary.

38. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins and antinutrients (e.g. protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems.

Appropriate oral toxicity studies[3] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, and taking into account its biological function in the plant where known.

39. Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the plant of the substance and dietary exposure. The types of studies to be performed may include studies on metabolism, toxicokinetics, subchronic toxicity, chronic toxicity/carcinogenicity, reproduction and development toxicity according to the traditional toxicological approach.

40. This may require the isolation of the new substance from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case, the material should be shown to be biochemically, structurally and functionally equivalent to that produced in the recombinant-DNA plant.

Assessment of possible allergenicity (proteins)

41. When the protein(s) resulting from the inserted gene is present in the food, it should be assessed for potential allergenicity in all cases. An integrated, stepwise, case-by-case approach used in the assessment of the potential allergenicity of the newly expressed protein(s) should rely upon various criteria used in combination (as no single criterion is sufficiently predictive on either allergenicity or non-allergenicity). As noted in paragraph 20, the data should be obtained using sound scientific methods. A detailed presentation of issues to be considered can be found in Annex 1 to this document.[4]

42. The newly expressed proteins in foods derived from recombinant-DNA plants should be evaluated for any possible role in the elicitation of gluten-sensitive enteropathy if the introduced genetic material is obtained from wheat, rye, barley, oats or related cereal grains.

43. The transfer of genes from commonly allergenic foods and from foods known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

Compositional analyses of key components

44. Analyses of concentrations of key components[5] of the recombinant-DNA plant and, especially those typical of the food, should be compared with an equivalent analysis

[3] Guidelines for oral toxicity studies have been developed in international fora, for example, the *OECD Guidelines for the Testing of Chemicals* issued by the Organisation for Economic Co-operation and Development.

[4] The FAO/WHO Expert Consultation 2001 report, which includes reference to several decision trees, was used in developing Annex 1 to these Guidelines.

[5] Key nutrients or key antinutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates as nutrients or enzyme inhibitors as antinutrients) or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased, selenium in wheat) and allergens.

of a conventional counterpart grown and harvested under the same conditions. In some cases, a further comparison with the recombinant-DNA plant grown under its expected agronomic conditions may need to be considered (e.g. application of a herbicide). The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. The comparator(s) used in this assessment should ideally be the near isogenic parental line. In practice, this may not be feasible at all times, in which case a line as close as possible should be chosen. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that are nutritionally important or that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

45. The location of trial sites should be representative of the range of environmental conditions under which the plant varieties would be expected to be grown. The number of trial sites should be sufficient to allow accurate assessment of compositional characteristics over this range. Similarly, trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature. To minimize environmental effects, and to reduce any effect from naturally occurring genotypic variation within a crop variety, each trial site should be replicated. An adequate number of plants should be sampled and the methods of analysis should be sufficiently sensitive and specific to detect variations in key components.

Evaluation of metabolites

46. Some recombinant-DNA plants may have been modified in a manner that could result in new or altered levels of various metabolites in the food. Consideration should be given to the potential for the accumulation of metabolites in the food that would adversely affect human health. Safety assessment of such plants requires investigation of residue and metabolite levels in the food and assessment of any alterations in nutrient profile. Where altered residue or metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g. procedures for assessing the human safety of chemicals in foods).

Food processing

47. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Therefore, information should be provided, describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.

Nutritional modification

48. The assessment of possible compositional changes to key nutrients, which should be conducted for all recombinant-DNA plants, has already been addressed under "Compositional analyses of key components". However, foods derived from

recombinant-DNA plants that have undergone modification to alter nutritional quality or functionality intentionally should be subjected to additional nutritional assessment in order to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply. A detailed presentation of issues to be considered can be found in Annex 2 to this document.

49. Information about the known patterns of use and consumption of a food, and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA plant. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups, such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.

50. The use of plant breeding, including *in vitro* nucleic acid techniques, to change nutrient levels in crops can result in broad changes to the nutrient profile in two ways. The intended modification in plant constituents could change the overall nutrient profile of the plant product, and this change could affect the nutritional status of individuals consuming the food. Unexpected alterations in nutrients could have the same effect. Although the recombinant-DNA plant components may be individually assessed as safe, the impact of the change on the overall nutrient profile should be determined.

51. When the modification results in a food product, such as vegetable oil, with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods or food components whose nutritional composition is closer to that of the food derived from recombinant-DNA plant) as appropriate comparators to assess the nutritional impact of the food.

52. Because of geographical and cultural variation in food consumption patterns, nutritional changes to a specific food may have a greater impact in some geographical areas or in some cultural populations than in others. Some food plants serve as the major source of a particular nutrient in some populations. The nutrient and the populations affected should be identified.

53. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. In addition, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization

of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.

SECTION 5 – OTHER CONSIDERATIONS

Potential accumulation of substances significant to human health

54. Some recombinant-DNA plants may exhibit traits (e.g. herbicide tolerance) that may indirectly result in the potential for accumulation of pesticide residues, altered metabolites of such residues, toxic metabolites, contaminants, or other substances that may be relevant to human health. The safety assessment should take this potential for accumulation into account. Conventional procedures for establishing the safety of such compounds (e.g. procedures for assessing the human safety of chemicals) should be applied.

Use of antibiotic resistance marker genes

55. Alternative transformation technologies that do not result in antibiotic resistance marker genes in foods should be used in the future development of recombinant-DNA plants, where such technologies are available and demonstrated to be safe.

56. Gene transfer from plants and their food products to gut micro-organisms or human cells is considered a rare possibility because of the many complex and unlikely events that would need to occur consecutively. Nevertheless, the possibility of such events cannot be completely discounted.[6]

57. In assessing the safety of foods containing antibiotic resistance marker genes, the following factors should be considered:

A. the clinical and veterinary use and importance of the antibiotic in question;
(Certain antibiotics are the only drug available to treat some clinical conditions (e.g. vancomycin for use in treating certain staphylococcal infections). Marker genes encoding resistance to such antibiotics should not be used in recombinant-DNA plants.)

B. whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficacy of the orally administered antibiotic; and
(This assessment should provide an estimate of the amount of orally ingested antibiotic that could be degraded by the presence of the enzyme in food, taking into account factors such as dosage of the antibiotic, amount of enzyme likely to remain in food following exposure to digestive conditions, including neutral or alkaline stomach conditions and the need for enzyme cofactors, e.g. adenosine triphosphate [ATP] for enzymatic activity and estimated concentration of such factors in food.)

[6] In cases where there are high levels of naturally occurring bacteria that are resistant to the antibiotic, the likelihood of such bacteria transferring this resistance to other bacteria will be orders of magnitude higher than the likelihood of transfer between ingested foods and bacteria.

C. safety of the gene product, as would be the case for any other expressed gene product.

58. If evaluation of the data and information suggests that the presence of the antibiotic resistance marker gene or gene product presents risks to human health, the marker gene or gene product should not be present in the food. Antibiotic resistance genes used in food production that encode resistance to clinically used antibiotics should not be present in foods.

Review of safety assessments

59. The goal of the safety assessment is a conclusion as to whether the new food is as safe as the conventional counterpart, taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

ANNEX 1

ASSESSMENT OF POSSIBLE ALLERGENICITY

SECTION 1 – INTRODUCTION

1. All newly expressed proteins[7] in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein. Therefore, it is recommended that an integrated, stepwise, case-by-case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data as no single criterion is sufficiently predictive.

3. The end-point of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

SECTION 2 – ASSESSMENT STRATEGY

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or acid and enzymatic treatment.

5. As there is no single test that can predict the likely human immunoglobulin E (IgE) response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA plant. Particular attention should be given to the choice of the expression host, as post-translational modifications allowed by different hosts (i.e.

[7] This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. The issue of enteropathies is already addressed in "Assessment of possible allergenicity (proteins)", paragraph 42 of the *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants*. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

eukaryotic vs prokaryotic systems) may have an impact on the allergenic potential of the protein.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

SECTION 3 – INITIAL ASSESSMENT

Section 3.1 – Source of the protein

7. As part of the data supporting the safety of foods derived from recombinant-DNA plants, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

Section 3.2 – Amino acid sequence homology

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results.[8] Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35 percent identity in a segment of 80 or more amino acids (FAO/WHO, 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically-based evaluation.

[8] It is recognized that the 2001 FAO/WHO Consultation suggested moving from 8 to 6 identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives; inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also Sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

Section 3.3 – Pepsin resistance

12. Resistance to pepsin digestion has been observed in several food allergens; thus, a correlation exists between resistance to digestion by pepsin and allergenic potential.[9] Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided.[10]

SECTION 4 – SPECIFIC SERUM SCREENING

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals.[11] In addition, the

[9] The method outlined in *The United States Pharmacopoeia* (1995) was used in the establishment of the correlation (Astwood *et al.*, 1996).

[10] Report of the Joint FAO/WHO Expert Consultation on the allergenicity of foods derived from biotechnology (2001): *Evaluation of allergenicity of genetically modified foods*, Section 6.4 Pepsin resistance.

[11] According to the report of the Joint FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology (22–25 January 2001, Rome) a minimum of eight relevant sera is required in order to achieve a 99-percent certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols.[12] A positive result in such tests would indicate a potential allergen.

SECTION 5 – OTHER CONSIDERATIONS

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute towards an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing that would be applied and its effects on the presence of the protein in the final food product.

17. As scientific knowledge and technology evolve, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include: targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

[12] *Ex vivo* procedure is described as the testing for allergenicity using cells or tissue culture from allergic human subjects (report of Joint FAO/WHO Expert Coosultation on allergenicity of foods derived from biotechnology.

ANNEX 2

FOOD SAFETY ASSESSMENT OF FOODS DERIVED FROM RECOMBINANT-DNA PLANTS MODIFIED FOR NUTRITIONAL OR HEALTH BENEFITS

SECTION 1 – INTRODUCTION

1. General guidance for the safety assessment of foods derived from recombinant-DNA plants is provided in the *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants* (CAC/GL 45-2003) (Plant Guideline). This Annex provides additional considerations that are specific to foods modified for nutritional or health benefits. The document does not extend beyond a safety assessment and, therefore, it does not cover assessment of the benefits themselves or any corresponding health claims, or risk-management measures.[13]

2. The following factors determine whether a recombinant-DNA plant is a recombinant-DNA plant modified for nutritional or health benefits, and as such within the scope of this Annex:
   a) the recombinant-DNA plant exhibits a particular trait in portion(s) of the plant intended for food use; and
   b) the trait is a result of: (i) introduction of a new nutrient(s) or related substance(s), (ii) alteration of either the quantity or bioavailability of a nutrient(s) or related substance(s), (iii) removal or reduction of undesirable substance(s) (e.g. allergens or toxicants), or (iv) alteration of the interaction(s) of nutritional or health relevance of these substances.

SECTION 2 – DEFINITION

3. The definition below applies to this Annex:

   Nutrient[14] means any substance normally consumed as a constituent of food:
   a) that provides energy; or
   b) that is needed for growth and development and maintenance of healthy life; or
   c) a deficit of which will cause characteristic biochemical or physiological changes to occur.

4. This Annex draws, where appropriate, on the definitions of key nutritional concepts to be found or to be developed in relevant Codex texts, especially those elaborated by the Codex Committee on Nutrition and Foods for Special Dietary Uses.

[13] *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003, paragraph 19).
[14] *General Principles for the addition of essential nutrients to foods* (CAC/GL 09–1987).

SECTION 3 – FOOD SAFETY ASSESSMENT

5. The *General Principles for the addition of essential nutrients to foods* (CAC/GL 09-1987) are generally applicable to the assessment of food derived from a plant that is modified by increasing the amount of a nutrient(s) or related substance(s) available for absorption and metabolism. The food safety framework outlined within the Plant Guideline[15] applies to the overall safety assessment of a food derived from a recombinant-DNA plant modified for nutritional or health benefits. This Annex presents additional considerations regarding the food safety assessment of those foods.

6. Foods derived from recombinant-DNA plants modified for nutritional or health benefits may benefit certain populations/subpopulations, while other populations/ subpopulations may be at risk from the same food.[16]

7. Rather than trying to identify every hazard associated with a particular food, the intention of a safety assessment of food derived from recombinant-DNA plants is the identification of new or altered hazards relative to the conventional counterpart.[17] As recombinant-DNA plants modified for nutritional or health benefits result in food products with a composition that may be significantly different from their conventional counterparts, the choice of an appropriate comparator[18] is of great importance for the safety assessment addressed in this Annex. Those alterations identified in a plant modified to obtain nutritional or health benefits are the subject of this safety assessment.

8. Upper levels of intake for many nutrients that have been set out by some national, regional and international bodies[19] may be considered, as appropriate. The basis for their derivation should also be considered in order to assess the public health implications of exceeding these levels.

9. The safety assessment of related substances should follow a case-by-case approach, taking into account upper levels as well as other values, where appropriate.

10. Although it is preferable to use a scientifically determined upper level of intake of a specific nutrient or related substance, when no such value has been determined, consideration may be given to an established history of safe use for nutrients or related substances that are consumed in the diet if the expected or foreseeable exposure would be consistent with those historical safe levels.

11. With conventional fortification of food, typically, a nutrient or a related substance is added at controlled concentrations and its chemical form is characterized. Levels of plant nutrients or related substances may vary in both conventionally bred and

[15] Paragraphs 18–21 and 48–53.
[16] Further guidance for susceptible and high-risk population groups is provided in paragraph 49 of the Plant Guideline.
[17] Plant Guideline, paragraph 4.
[18] Plant Guideline, paragraph 51.
[19] Where such guidance is not provided by Codex, information provided by FAO/WHO may be preferably considered.

recombinant-DNA plants owing to growing conditions. In addition, more than one chemical form of the nutrient might be expressed in the food as a result of the modification and these may not be characterized from a nutrition perspective. Where appropriate, information may be needed on the different chemical forms of the nutrient(s) or related substance(s) expressed in the portion of the plant intended for food use and their respective levels.

12. Bioavailability of the nutrient(s), related substance(s) or undesirable substance(s) in the food that were the subject of the modification in the recombinant-DNA plant should be established, where appropriate. If more than one chemical form of the nutrient(s) or related substance(s) is present, their combined bioavailability should be established, where appropriate.

13. Bioavailability will vary for different nutrients, and methods of testing for bioavailability should be relevant to the nutrient and the food containing the nutrient, as well as the health, nutritional status and dietary practices of the specific populations consuming the food. *In vitro* and *in vivo* methods to determine bioavailability exist, the latter conducted in animals and in humans. *In vitro* methods can provide information to assess extent of release of a substance from plant tissues during the digestive process. *In vivo* studies in animals are of limited value in assessing nutritional value or nutrient bioavailability for humans and would require careful design in order to be relevant. *In vivo* studies, in particular, human studies, may provide more relevant information about whether and to what extent the nutrient or related substance is bioavailable.

14. Guidance on dietary exposure assessment of foods derived from recombinant-DNA plants with nutritional modifications is provided in paragraph 49 of the Plant Guideline. In the context of this Annex, dietary exposure assessment is the estimation of the concentration of the nutrient(s) or related substance(s) in a food, the expected or foreseeable consumption of that food, and any known factors that influence bioavailability. Exposure to a nutrient(s) or related substance(s) should be evaluated in the context of the total diet and the assessment should be carried out based on the customary dietary consumption by the relevant population(s) of the corresponding food that is likely to be displaced. When evaluating the exposure, it is appropriate to consider information on whether the consumption of the modified food could lead to adverse nutritional effects as compared with consumption of the food that it is intended to replace. Most, if not all, aspects of exposure assessment are not unique to recombinant-DNA plants modified for nutritional or health benefits.[20]

15. The first step of an exposure assessment is determining the level(s) of the substance(s) in question in the portion of the plant intended for food use. Guidance on determining changes in levels of these substances is provided in the Plant Guideline.[21]

[20] Additional applicable guidance on dietary exposure assessment of nutrients and related substances is provided in the report of the Joint FAO/WHO Technical Workshop on nutrient risk assessment: *A model for establishing upper levels of intake for nutrients and related substances*, WHO Headquarters, Geneva, Switzerland, 2–6 May 2005.

[21] Paragraphs 44 and 45.

16. Consumption patterns will vary from country to country depending on the importance of the food in the diet(s) of a given population(s). Therefore, it is recommended that consumption estimates are based on national or regional food consumption data when available, using existing guidance on estimation of exposure in a given population(s).[22] When national or regional food consumption data are unavailable, food availability data may provide a useful resource.[23]

17. To assess the safety of a food derived from a recombinant-DNA plant modified for a nutritional or health benefit, the estimated intake of the nutrient or related substance in the population(s) is compared with the nutritional or toxicological reference values, such as upper levels of intake, acceptable daily intakes (ADIs) for that nutrient or related substance, where these values exist. This may involve assessments of different consumption scenarios against the relevant nutritional reference value, taking into account possible changes in bioavailability, or extend to probabilistic methods that characterize the distribution of exposures within the relevant population(s).

[22] *A model for establishing upper levels of intake for nutrients and related substances*. Report of the Joint FAO/WHO Technical Workshop on nutrient risk assessment. WHO Headquarters, Geneva, Switzerland, 2–6 May 2005.

[23] Data on staple food products may also be supplemented by information from FAO Food Balance Sheets.

ANNEX 3

FOOD SAFETY ASSESSMENT IN SITUATIONS OF LOW-LEVEL PRESENCE OF RECOMBINANT-DNA PLANT MATERIAL IN FOOD

SECTION 1 – PREAMBLE

1. An increasing number of recombinant-DNA plants are being authorized for commercialization. However, they are authorized at different rates in different countries. As a consequence of these asymmetric authorizations, low levels of recombinant-DNA plant materials that have passed a food safety assessment according to the *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants* (CAC/GL 45-2003) (Plant Guideline) in one or more countries may on occasion be present in food in importing countries in which the food safety of the relevant recombinant-DNA plants has not been determined.

2. This Annex describes the recommended approach to the food safety assessment in such situations of low-level presence of recombinant-DNA plant material or in advance preparation for such potential circumstances.[24]

3. This Annex also describes data- and information-sharing mechanisms to facilitate utilization of the Annex and to determine whether it should apply.

4. This Annex can be applied in two different dietary exposure situations:
   a) That involving commodities, such as grains, beans or oilseeds, in which exposure to food from a variety not authorized in the importing country would likely be to dilute low-level amounts at any one time. This would probably be the more common situation of low-level presence of recombinant-DNA plant material. Because any food-serving of grains, beans or oilseeds would almost necessarily come from multiple plants, and because of how these types of commodities generally are sourced from multiple farms, are commingled in grain elevators, are further commingled in export shipments, at import and when used in processed foods, any inadvertently commingled material derived from recombinant-DNA plant varieties would be present only at a low level in any individual serving of food.
   b) That involving foods that are commonly consumed whole and undiluted, such as some fruits and vegetables like potatoes, tomatoes, and papaya, in which exposure would be rare but could be to an undiluted form of the unauthorized recombinant-DNA plant material. While the likelihood of consuming material from such an unauthorized variety would be low and the likelihood of repeated consumption would be much lower, any such consumption might be of an entire unauthorized fruit or vegetable.

[24] This guidance is not intended for a recombinant-DNA plant that was not authorized in an importing country as a result of food safety assessment by that country.

5. In both cases, the dietary exposure will be significantly lower than would be considered in a food safety assessment of the recombinant-DNA plant according to the Plant Guideline. As a result, only certain elements of the Plant Guideline will be relevant and, therefore, are included in this Annex.

6. This Annex does not:
- address risk management measures; national authorities will determine when a recombinant-DNA plant material is present at a level low enough for this Annex to be appropriate;
- preclude national authorities from conducting a safety assessment according to the Plant Guideline; countries can decide when and how to use the Annex within the context of their regulatory systems; or
- eliminate the responsibility of industries, exporters and, when applicable, national competent authorities to continue to meet the relevant import requirements set by countries, including in relation to unauthorized recombinant-DNA plant material.

SECTION 2 – GENERAL AND OTHER CONSIDERATIONS

7. For the food safety assessment in situations of low-level presence of recombinant DNA plant materials in food, Sections 4 and 5 of the Plant Guideline apply as amended as follows. The applicable paragraphs are specifically indicated. Those paragraphs of the Plant Guidelines that are not listed can be omitted from consideration.

Description of the recombinant-DNA plant

8. Paragraph 22 of the Plant Guideline applies.

Description of the host plant and its use as a food

9. Paragraphs 23, 24 and 25 of the Plant Guideline apply.

Description of the donor organism(s)

10. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. The description of the donor organism(s) should include:

A. its usual or common name;
B. scientific name;
C. taxonomic classification;
D. information about the natural history as concerns food safety;
E. information on naturally occurring toxins and allergens; for micro-organisms, additional information on pathogenicity and the relationship to known pathogens; and

F. information on past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g. possible presence as contaminants).[25]

Description of the genetic modification(s)

11. Paragraphs 27, 28 and 29 of the Plant Guideline apply.

Characterization of the genetic modification(s)

12. Paragraphs 30 and 31 of the Plant Guideline apply.

13. Information should be provided on any expressed substances in the recombinant-DNA plant; this should include:
   A. the gene product(s) (e.g. a protein or an untranslated RNA);
   B. the function of the gene product(s);
   C. the phenotypic description of the new trait(s);
   D. the level and site of expression in the plant of the expressed gene product(s), and the levels of its metabolites in the edible portions of the plant; and
   E. where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous mRNA or protein.[26]

14. Paragraph 33 of the Plant Guideline applies.

Safety assessment

Expressed substances (non-nucleic acid substances)

Assessment of possible toxicity

15. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations and mean values.[27]

16. Information should be provided to ensure that genes coding for known toxins present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, as conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate toxicants.[28]

17. Paragraph 37 of the Plant Guideline applies.

18. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins as well as stability to heat or processing and to degradation in appropriate representative gastric and

[25] The text of this paragraph was adapted from paragraph 26 of the Plant Guideline.
[26] The text of this paragraph was adapted from paragraph 32 of the Plant Guideline.
[27] The text of this paragraph was adapted from paragraph 35 of the Plant Guideline.
[28] The text of this paragraph was adapted from paragraph 36 of the Plant Guideline.

intestinal model systems. Appropriate oral toxicity studies[29] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, and taking into account its biological function in the plant where known.[30]

19. Paragraphs 39 and 40 of the Plant Guideline apply.

Assessment of possible allergenicity (proteins)

20. Paragraphs 41, 42 and 43 of the Plant Guideline apply.

Analyses of key toxicants and allergens

21. Analyses of key toxicants[31] and allergens are important in certain cases of foods from recombinant-DNA plants (e.g. those that are commonly consumed whole and undiluted, such as potatoes, tomatoes, and papaya). Analyses of concentrations of key toxicants and allergens of the recombinant-DNA plant typical of the food should be compared with an equivalent analysis of a conventional counterpart grown and harvested under the same conditions. The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. The comparator(s) used in this assessment should ideally be the near isogenic parental line. In practice, this may not be feasible at all times, in which case a line as close as possible should be chosen. The purpose of this comparison is to establish that substances that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.[32]

22. The location of trial sites should be representative of the range of environmental conditions under which the plant varieties would be expected to be grown. The number of trial sites should be sufficient to allow accurate assessment of key toxicants and allergens over this range. Similarly, trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature. To minimize environmental effects, and to reduce any effect from naturally occurring genotypic variation within a crop variety, each trial site should be replicated. An adequate number of plants should be sampled and the methods of analysis should be sufficiently sensitive and specific to detect variations in key toxicants and allergens.[33]

Evaluation of metabolites

23. Some recombinant-DNA plants may have been modified in a manner that could result in new or altered levels of various metabolites in the food. In certain cases of foods from recombinant-DNA plants (e.g. those that are commonly consumed whole

[29] Guidelines for oral toxicity studies have been developed in international fora, for example, the *OECD Guidelines for the Testing of Chemicals* issued by the Organisation for Economic Co-operation and Development.

[30] The text of this paragraph was adapted from paragraph 38 of the Plant Guideline.

[31] Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased).

[32] The text of this paragraph was adapted from paragraph 44 of the Plant Guideline.

[33] The text of this paragraph was adapted from paragraph 45 of the Plant Guideline.

and undiluted), consideration should be given to the potential for the accumulation of metabolites in the food that would adversely affect human health. Food safety assessment in situations of low-level presence of recombinant-DNA material in foods from such plants requires investigation of residue and metabolite levels in the food. Where altered residue or metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g. procedures for assessing the human safety of chemicals in foods).[34]

Food processing

24. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant. Therefore, information should be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.[35]

Potential accumulation of substances significant to human health

25. Some recombinant-DNA plants may exhibit traits (e.g. herbicide tolerance) that may indirectly result in the potential for accumulation of pesticide residues, altered metabolites of such residues, toxic metabolites, contaminants, or other substances that may be relevant to human health. In certain cases of foods from recombinant-DNA plants (e.g. those that are commonly consumed whole and undiluted), the risk assessment should take this potential for accumulation into account. Conventional procedures for establishing the safety of such compounds (e.g. procedures for assessing the human safety of chemicals) should be applied.[36]

Use of antibiotic resistance marker genes

26. Paragraphs 55, 56, 57 and 58 of the Plant Guideline apply.

SECTION 3 – GUIDANCE ON DATA AND INFORMATION SHARING

27. In order for Codex Members to use this Annex, it is essential that they have access to requisite data and information.

28. Codex Members should make available to a publicly accessible central database to be maintained by FAO information on recombinant-DNA plants authorized in accordance with the Plant Guideline. This information should be presented in accordance with the following format:
    a) name of product applicant;
    b) summary of application;
    c) country of authorization;

[34] The text of this paragraph was adapted from paragraph 46 of the Plant Guideline.
[35] The text of this paragraph was adapted from paragraph 47 of the Plant Guideline.
[36] The text of this paragraph was adapted from paragraph 54 of the Plant Guideline.

d) date of authorization;
e) scope of authorization;
f) unique identifier;
g) links to the information on the same product in other databases maintained by relevant international organizations, as appropriate;
h) summary of the safety assessment, which should be consistent with the framework of food safety assessment of the Plant Guideline;
i) where detection method protocols and appropriate reference material (non-viable or, in certain circumstances, viable) suitable for low-level situation may be obtained[37]; and
j) contact details of the competent authority(s) responsible for the safety assessment and the product applicant.

29. This process should facilitate rapid access by importing Codex Members to additional information relevant to the assessment of food safety assessment in situations of low-level presence of recombinant-DNA plant material in foods in accordance with this Annex.

30. The authorizing Codex Members should make available complementary information to other Codex Members on its safety assessment in accordance with the Plant Guideline, in conformity with its regulatory/legal framework.

31. The product applicant should provide further information and clarification as necessary to allow the assessment according to this Annex to proceed, as well as a validated protocol for an event-specific or trait-specific detection method suitable for low-level situations and appropriate reference materials (non-viable or, in certain circumstances, viable). This is without prejudice to legitimate concerns to safeguard the confidentiality of commercial and industrial information.

32. As appropriate, new scientific information relevant to the conclusions of the food safety assessment conducted in accordance with the Plant Guideline by the authorizing Codex Member should be made available.

[37] This information may be provided by the product applicant or in some cases by Codex Members.

GUIDELINE FOR THE CONDUCT OF FOOD SAFETY ASSESSMENT OF FOODS PRODUCED USING RECOMBINANT-DNA MICRO-ORGANISMS

CAC/GL 46-2003

SECTION 1 – SCOPE

1. This Guideline supports the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003) and addresses safety and nutritional aspects of foods produced through the actions of recombinant deoxyribonucleic acid (recombinant-DNA) micro-organisms.[1] The recombinant-DNA micro-organisms that are used to produce these foods are typically derived using the techniques of modern biotechnology from strains that have a history of safe, purposeful use in food production. However, in instances where the recipient strains do not have a history of safe use, their safety will have to be established.[2] Such food and food ingredients may contain viable or non-viable recombinant-DNA micro-organisms or may be produced by fermentation using recombinant-DNA micro-organisms from which the recombinant-DNA micro-organisms may have been removed.

2. Recognizing that the following issues may have to be addressed by other bodies or other instruments, this document does not address:
   - safety of micro-organisms used in agriculture (for plant protection, biofertilizers, in animal feed or food derived from animals fed the feed, etc.);
   - risks related to environmental releases of recombinant-DNA micro-organisms used in food production;
   - safety of substances produced by micro-organisms that are used as additives or processing aids, including enzymes for use in food production;[3]
   - specific purported health benefits or probiotic effects that may be attributed to the use of micro-organisms in food; or
   - issues relating to the safety of food production workers handling recombinant-DNA micro-organisms.

3. A variety of micro-organisms used in food production have a long history of safe use that predates scientific assessment. Few micro-organisms have been assessed scientifically in a manner that would fully characterize all potential risks associated with the food they are used to produce, including, in some instances, the consumption of viable micro-

[1] The micro-organisms included in these applications are bacteria, yeasts and filamentous fungi. (Such uses could include, but are not limited to, production of yogurt, cheese, fermented sausages, natto, kimchi, bread, beer and wine.)

[2] The criterion for establishing the safety of micro-organisms used in the production of foods where there is no history of safe use is beyond the scope of the current document.

[3] The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is revising guidelines for *General specifications and considerations for enzyme preparations used in food processing*. These guidelines have been used to evaluate enzyme preparations derived from genetically modified micro-organisms.

organisms. Furthermore, the Codex Principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities such as food additives and pesticide residues, or specific chemical or microbial contaminants that have identifiable hazards and risks; they were not originally intended to apply to intentional uses of micro-organisms in food processing or in the foods transformed by microbial fermentations. The safety assessments that have been conducted have focused primarily on the absence of properties associated with pathogenicity in these micro-organisms and the absence of reports of adverse events attributed to ingestion of these micro-organisms, rather than evaluating the results of prescribed studies. Further, many foods contain substances that would be considered harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. Information considered in developing this approach includes:
   A. uses of living micro-organisms in food production;
   B. consideration of the types of genetic modifications likely to have been made in these organisms;
   C. the types of methodologies available for performing a safety assessment; and
   D. issues specific to the use of the recombinant-DNA micro-organism in food production, including its genetic stability, potential for gene transfer, colonization of the gastrointestinal tract and persistence[4] therein, interactions that the recombinant-DNA micro-organism may have with the gastrointestinal flora or the mammalian host, and any impact of the recombinant-DNA micro-organism on the immune system.

5. This approach is based on the principle that the safety of foods produced using recombinant-DNA micro-organisms is assessed relative to the conventional counterparts that have a history of safe use, not only for the food produced using a recombinant-DNA micro-organism, but also for the micro-organism itself. This approach takes both intended and unintended effects into account. Rather than trying to identify every hazard associated with a particular food or the micro-organism, the intention is to identify new or altered hazards relative to the conventional counterpart.

6. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003). If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and, if necessary, further risk assessment, the food or component of food, such as a micro-organism used in production, would be subjected to risk management considerations in accordance with the *Principles for the risk analysis of foods derived*

[4] Persistence connotes survival of micro-organisms in the gastrointestinal tract longer than two intestinal transit times (International Life Science Institute, *The safety assessment of viable genetically modified micro-organisms used as food*, 1999, Brussels; the Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, *Safety assessment of foods derived from genetically modified micro-organisms*, 24–28 September 2001, Geneva, Switzerland).

from modern biotechnology (CAC/GL 44-2003) before it is considered for commercial distribution.

7. Risk management measures, such as post-market monitoring of consumer health effects, may assist the risk assessment process. These are discussed in paragraph 20 of the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003).

8. The Guideline describes approaches recommended for making safety assessments of foods produced using recombinant-DNA micro-organisms, using comparison with a conventional counterpart. The safety assessment will focus on the safety of the recombinant-DNA micro-organisms used in food production and, where appropriate, on metabolites produced by the action of recombinant-DNA micro-organisms on food. The Guideline identifies the data and information that are generally applicable to making such assessments. When conducting a comparison of a recombinant-DNA micro-organism or a food produced using recombinant-DNA micro-organism with their respective conventional counterparts, any identified differences should be taken into account, whether they are the result of intended or unintended effects. Due consideration should be given to the interactions of the recombinant-DNA micro-organism with the food matrix or the microflora and to the safety of any newly expressed protein(s) and secondary metabolic products. While this Guideline is designed for foods produced using recombinant-DNA micro-organisms or their components, the approach described could, in general, be applied to foods produced using micro-organisms that have been altered by other techniques.

SECTION 2 – DEFINITIONS

9. The definitions below apply to this Guideline:

Recombinant-DNA micro-organism means bacteria, yeasts or filamentous fungi in which the genetic material has been changed through *in vitro* nucleic acid techniques including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

Conventional counterpart[5] means:

- a micro-organism/strain with a known history of safe use in producing and/or processing the food and related to the recombinant-DNA strain. The micro-organism may be viable in the food or may be removed in processing or rendered non-viable during processing; or
- food produced using the traditional food production micro-organisms for which there is experience of establishing safety based on common use in food production.

[5] It is recognized that, for the foreseeable future, micro-organisms derived from modern biotechnology will not be used as conventional counterparts.

SECTION 3 – INTRODUCTION TO FOOD SAFETY ASSESSMENT

10. Most foods produced as a result of the purposeful growth of micro-organisms have their origins in antiquity, and have been deemed safe since long before the emergence of scientific methods for assessing safety. Micro-organisms possess properties, such as fast growth rates, that enable genetic modifications, whether employing conventional techniques or modern biotechnology, to be implemented in short time frames. Micro-organisms used in food production derived using conventional genetic techniques have not customarily been systematically subjected to extensive chemical, toxicological, epidemiological or medical evaluations prior to marketing. Instead, microbiologists, mycologists and food technologists have evaluated new strains of bacteria, yeasts and filamentous fungi for phenotypic characteristics that are useful in relation to food production.

11. Safety assessments of recombinant-DNA micro-organisms should document the use of related micro-organisms in foods, the absence of properties known to be characteristic of pathogens in the recombinant-DNA micro-organisms or the recipient strains used for constructing the recombinant-DNA micro-organisms, and known adverse events involving the recipient or related organisms. In addition, when a recombinant DNA micro-organism directly affects or remains in the food, any effects on the safety of the food should be examined.

12. The use of animal models for assessing toxicological effects is a major element in the risk assessment of many compounds, such as pesticides. However, in most cases, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. Therefore, it is relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

13. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds and often characterized by a wide variation in composition and nutritional value. Owing to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects that are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can, therefore, be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

14. Animal studies typically employed in toxicological evaluations also cannot be readily applied to testing potential risks associated with ingestion of micro-organisms used for food production. Micro-organisms are living entities, containing complex structures composed of many biochemicals and, therefore, are not comparable with pure compounds. In some processed foods, they can survive processing and ingestion and can compete and, in some cases, be retained in the intestinal environment for significant periods of time. Appropriate animal studies should be used to evaluate the safety of recombinant-DNA micro-organisms where the donor or the gene or gene product do not have a history of safe use in food, taking into account available information regarding the donor and the characterization of the modified genetic material and the gene product. Further, appropriately designed studies in animals may be used to assess the nutritional value of the food or the bioavailability of the newly expressed substance in the food.

15. Owing to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods produced using recombinant-DNA micro-organisms. This has been addressed by the development of a multidisciplinary approach for assessing safety that takes into account the intended effect, the nature of the modification and detectable unintended changes that may occur in the micro-organism or in its action on the food, using the concept of substantial equivalence.[6]

16. While the focus of a safety assessment will be on the recombinant-DNA micro-organism, additional information on its interaction with the food matrix should be taken into consideration when applying the concept of substantial equivalence, which is a key step in the safety assessment process. However, the concept of substantial equivalence is not a safety assessment in itself. Rather it represents the starting point that is used to structure the safety assessment of both a recombinant-DNA micro-organism relative to its conventional counterpart and the food produced using a recombinant-DNA micro-organism relative to its conventional counterpart. This concept is used to identify for evaluation similarities and differences between recombinant-DNA micro-organisms used in food processing as well as the food produced using the recombinant-DNA micro-organisms and their respective conventional counterparts as defined in paragraph 9. It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods produced using recombinant-DNA micro-organisms. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the recombinant-DNA micro-organism and the food produced using the recombinant-DNA micro-organism can be considered relative to their respective conventional counterparts.

[6] The concept of *substantial equivalence* as described in the Joint FAO/WHO Expert Consultation on foods derived from biotechnology, *Safety aspects of genetically modified plants*, 29 May – 2 June 2000, Geneva, Switzerland, and Section 4.3 of the Joint FAO/WHO Expert Consultation of Foods Derived from Biotechnology, *Safety assessment of foods derived from genetically modified micro-organisms*, 24–28 September 2001, Geneva, Switzerland.

Unintended effects

17. In achieving the objective of conferring a specific target trait (intended effect) to a micro-organism by the addition, substitution, removal or rearrangement of defined DNA sequences, including those used for the purpose of DNA transfer or maintenance in the recipient organism, additional traits could, in some cases, be acquired or existing traits could be lost or modified. The potential for occurrence of unintended effects is not restricted to the use of *in vitro* nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in the development of strains using traditional genetic techniques and procedures, or from exposure of micro-organisms to intentional or unintended selective pressures. Unintended effects may be deleterious, beneficial or neutral with respect to competition with other micro-organisms, ecological fitness of the micro-organism, the effects of the micro-organism on humans after ingestion, or the safety of foods produced using the micro-organism. Unintended effects in recombinant-DNA micro-organisms may also arise through intentional modification of DNA sequences or they may arise through recombination or other natural events in the recombinant-DNA micro-organism. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA micro-organism would have an unexpected, adverse effect on human health.

18. Unintended effects can result from the insertion of DNA sequences new to a micro-organism into the microbial genome; they may be compared with those observed following the activity of naturally occurring transposable genetic elements. Insertion of DNA may lead to changes in expression of genes in the genome of the recipient. The insertion of DNA from heterologous sources into a gene may also result in the synthesis of a chimeric protein, also referred to as a fusion protein. In addition, genetic instability and its consequences need to be considered.

19. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels or the expression of an enzyme new to the organism may give rise to secondary biochemical effects, changes in the regulation of metabolic pathways or altered levels of metabolites.

20. Unintended effects caused by to genetic modification may be subdivided into two groups: those that could be predicted, and those that are "unexpected". Many unintended effects are largely predictable based on knowledge of the added trait, its metabolic consequences or the site of insertion. As a consequence of the expanding knowledge of microbial genomes and physiology, and the increased specificity in function of genetic materials introduced through recombinant-DNA techniques compared with other forms of genetic manipulation, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse changes that occur at the level of transcription and translation that could lead to unintended effects.

21. The safety assessment of foods produced using recombinant-DNA micro-organisms involves methods to identify and detect such unintended effects, and procedures to evaluate their biological relevance and potential impact on food safety. A variety of

data and information is necessary to assess unintended effects, because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, should provide assurance that the food is unlikely to have an adverse effect on human health. The assessment of unintended effects takes into account the biochemical and physiological characteristics of the micro-organism that are typically selected for improving strains for commercial food or beverage uses. These determinations provide a first screen for micro-organisms that exhibit unintended traits. Recombinant-DNA micro-organisms that pass this screen are subjected to safety assessment as described in Section 4.

Framework of food safety assessment

22. The safety assessment of a food produced using a recombinant-DNA micro-organism is based on determining the safety of using the micro-organism. It follows a stepwise process of addressing relevant factors, which include:

A. description of the recombinant-DNA micro-organism;
B. description of the recipient micro-organism and its use in food production;
C. description of the donor organism(s);
D. description of the genetic modification(s) including vector and construct;
E. characterization of the genetic modification(s);
F. safety assessment:
   a) expressed substances: assessment of potential toxicity and other traits related to pathogenicity,
   b) compositional analyses of key components,
   c) evaluation of metabolites,
   d) effects of food processing,
   e) assessment of immunological effects,
   f) assessment of viability and residence of micro-organisms in the human gastrointestinal tract,
   g) antibiotic resistance and gene transfer, and
   h) nutritional modification.

23. In certain cases, the characteristics of the micro-organisms and/or the foods produced/processed using these micro-organisms may necessitate generation of additional data and information to address issues that are unique to the micro-organisms and/or food products under review.

24. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, good laboratory practice. Primary data should be made available to regulatory authorities upon request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

25. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food will not cause harm when prepared or consumed according to its intended use, nor should the organism itself cause harm

when viable organisms remain in the food. Safety assessments should address the health aspects for the whole population, including immuno-compromised individuals, infants and the elderly. The expected end-point of such an assessment will be a conclusion regarding whether the new food and/or micro-organisms are as safe as the conventional counterparts, taking into account dietary impact of any changes in nutritional content or value. Where the micro-organism is likely to be viable upon ingestion, its safety should be compared with a conventional counterpart, taking into account residence of the recombinant-DNA micro-organism in the gastrointestinal tract, and where appropriate, interactions between it and the gastrointestinal flora of mammals (especially humans) and impacts of the recombinant-DNA micro-organism on the immune system. In essence, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed to protect the health of consumers and, if so, to make well-informed and appropriate decisions in this regard.

SECTION 4 – GENERAL CONSIDERATIONS

Description of the recombinant-DNA micro-organism

26. A description of the bacterial, yeast or fungal strain and the food being presented for safety assessment should be provided. This description should be sufficient to aid in understanding the nature of the organism or food produced using the organism being submitted for safety assessment. Recombinant-DNA micro-organisms used in food production or contained in food should be conserved as stock cultures with appropriate identification using molecular methods, and preferably, in established culture collections. This may facilitate the review of the original safety assessment. Such stock cultures should be made available to regulatory authorities upon request.

Description of the recipient micro-organism and its use in food production

27. A comprehensive description of the recipient micro-organism or micro-organism subjected to the modification should be provided. Recipient micro-organisms should have a history of safe use in food production or safe consumption in foods. Organisms that produce toxins, antibiotics or other substances that should not be present in food, or that bear genetic elements that could lead to genetic instability, antibiotic resistance, or that are likely to contain genes conferring functions associated with pathogenicity (i.e. also known as pathogenicity islands or virulence factors) should not be considered for use as recipients. The necessary data and information should include, but need not be restricted to:

A. identity: scientific name, common name or other name(s) used to reference the micro-organism, strain designation, information about the strain and its source, or accession numbers or other information from a recognized culture repository from which the organism or its antecedents may be obtained, if applicable, and information supporting its taxonomical assignment;

B. history of use and cultivation, known information about strain development (including isolation of mutations or antecedent strains used in strain construction); in particular, identifying traits that may adversely affect human health;

C. information on the genotype and phenotype of the recipient micro-organism relevant to its safety, including any known toxins, antibiotics, antibiotic resistance factors or other factors related to pathogenicity, or immunological impact, and information about the genetic stability of the micro-organism;
D. history of safe use in food production or safe consumption in food; and
E. information on the relevant production parameters used to culture the recipient micro-organism.

28. Relevant phenotypic and genotypic information should be provided not only for the recipient micro-organism, but also for related species and for any extra-chromosomal genetic elements that contribute to the functions of the recipient strain, particularly if the related species are used in foods or involved in pathogenic effects in humans or other animals. Information on the genetic stability of the recipient micro-organism should be considered including, as appropriate, the presence of mobile DNA elements, i.e. insertion sequences, transposons, plasmids and prophages.

29. The history of use may include information on how the recipient micro-organism is typically grown, transported and stored, quality assurance measures typically employed, including those to verify strain identity and production specifications for micro-organisms and foods, and whether these organisms remain viable in the processed food or are removed or rendered non-viable as a consequence of processing.

Description of the donor organism(s)

30. Information should be provided on the donor organism(s) and any intermediate organisms, when applicable, and, when relevant, related organisms. It is particularly important to determine if the donor or intermediate organism(s) or other closely related species naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. The description of the donor or intermediate organism(s) should include:

A. identity: scientific name, common name or other name(s) used to reference the organism, strain designation, information about the strain and its source, or accession numbers or other information from a recognized culture repository from which the organism or its antecedents may be obtained, if applicable, and information supporting its taxonomic assignment;
B. information about the organism or related organisms that concerns food safety;
C. information on the genotype and phenotype of the organism relevant to its safety including any known toxins, antibiotics, antibiotic resistance factors or other factors related to pathogenicity, or immunological impact; and
D. information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g. possible presence as contaminants).

Description of the genetic modification(s) including vector and construct

31. Sufficient information should be provided on the genetic modification(s) to allow for the identification of all genetic material potentially delivered to or modified in the recipient micro-organism and to provide the necessary information for the analysis of

the data supporting the characterization of the DNA added to, inserted into, modified in or deleted from the microbial genome.

32. The description of the strain construction process should include:
   A. information on the specific method(s) used for genetic modification;
   B. information on the DNA used to modify the micro-organism, including the source (e.g. plant, microbial, viral, synthetic), identity and expected function in the recombinant-DNA micro-organism, and copy number for plasmids; and
   C. intermediate recipient organisms including the organisms (e.g. other bacteria or fungi) used to produce or process DNA prior to introduction into the final recipient organism.

33. Information should be provided on the DNA added, inserted, deleted or modified, including:
   A. the characterization of all genetic components, including marker genes, vector genes, regulatory and other elements affecting the function of the DNA;
   B. the size and identity;
   C. the location and orientation of the sequence in the final vector/construct; and
   D. the function.

Characterization of the genetic modification(s)

34. In order to provide clear understanding of the impact of the genetic modification on the composition and safety of foods produced using recombinant-DNA micro-organisms, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out. To facilitate the safety assessment, the DNA to be inserted should preferably be limited to the sequences necessary to perform the intended functions.

35. Information should be provided on the DNA modifications in the recombinant DNA micro-organism; this should include:
   A. the characterization and description of the added, inserted, deleted or otherwise modified genetic materials, including plasmids or other carrier DNA used to transfer desired genetic sequences. This should include an analysis of the potential for mobilization of any plasmids or other genetic elements used, the locations of the added, inserted, deleted or otherwise modified genetic materials (site on a chromosomal or extra-chromosomal location); if located on a multicopy plasmid, the copy number of the plasmid;
   B. the number of insertion sites;
   C. the organization of the modified genetic material at each insertion site. including the copy number and sequence data of the inserted, modified or deleted material, plasmids or carrier DNA used to transfer the desired genetic sequences, and the surrounding sequences. This will enable the identification of any substances expressed as a consequence of the inserted, modified or deleted material;
   D. identification of any open reading frames within inserted DNA or created by the modifications to contiguous DNA in the chromosome or in a plasmid, including those that could result in fusion proteins; and

E. particular reference to any sequences known to encode, or to influence the expression of, potentially harmful functions.

36. Information should be provided on any expressed substances in the recombinant-DNA micro-organism; this should include:

A. the gene product(s) (e.g. a protein or an untranslated ribonucleic acid [RNA]) or other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food;
B. the function of the gene product;
C. the phenotypic description of the new trait(s);
D. the level and site of expression (intracellular, periplasmic – for Gram-negative bacteria, organellar – in eukaryotic micro-organisms, secreted) in the micro-organism of the expressed gene product(s), and, when applicable, the levels of its metabolites in the organism;
E. the amount of the inserted gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the level of a specific endogenous messenger RNA (mRNA) or protein; and
F. the absence of a gene product, or alterations in metabolites related to gene products, if applicable to the intended function(s) of the genetic modification(s).

37. In addition, information should be provided:

A. to demonstrate whether the arrangement of the modified genetic material has been conserved[7] or whether significant rearrangements have occurred after introduction to the cell and propagation of the recombinant strain to the extent needed for its use(s) in food production, including those that may occur during its storage according to current techniques;
B. to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;
C. to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable for the extent of propagation needed for its use(s) in food production and is consistent with laws of inheritance. It may be necessary to examine the inheritance of the inserted or modified DNA or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly;[8]
D. to demonstrate whether the newly expressed trait(s) is expressed as expected and targeted to the appropriate cellular location or is secreted in a manner and at levels that is consistent with the associated regulatory sequences driving the expression of the corresponding gene;

[7] Microbial genomes are more fluid than those of higher eukaryotes; that is, the organisms grow faster, adapt to changing environments, and are more prone to change. Chromosomal rearrangements are common. The general genetic plasticity of micro-organisms may affect recombinant DNA in micro-organisms and must be considered in evaluating the stability of recombinant DNA micro-organisms.

[8] Modified strains should be maintained in a manner to enable verification of the genetic stability.

E. to indicate whether there is any evidence to suggest that one or more genes in the recipient micro-organism have been affected by the modifications or the genetic exchange process; and
F. to confirm the identity and expression pattern of any new fusion proteins.

Safety assessment

38. The safety assessment of the modified micro-organism should be performed on a case-by-case basis depending on the nature and extent of the introduced changes. Conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary. Effects of the recombinant-DNA micro-organism on the food matrix should be considered as well. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal or *in vitro* studies with the recombinant-DNA micro-organism and/or the food produced using it could be considered necessary.

Expressed substances: assessment of potential toxicity and other traits related to pathogenicity

39. When a substance is new to foods or food processing, the use of conventional toxicology studies or other applicable studies on the new substance will be necessary. This may require the isolation of the new substance from the recombinant-DNA micro-organism, the food product if the substance is secreted, or, if necessary, the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA micro-organism. Information on the anticipated exposure of consumers to the substance, and on the potential intake and dietary impact of the substance should be provided.

40. The safety assessment of the expressed substance should take into account its function and concentration in the food. The number of viable micro-organisms remaining in the food should be also determined and compared with a conventional counterpart. All quantitative measurements should be analysed using appropriate statistical techniques. Current dietary exposure and possible effects on population subgroups should also be considered.

- In the case of proteins, the assessment of potential toxicity should take into account the structure and function of the protein and should focus on amino acid sequence similarity between the protein and known protein toxins and antinutrients (e.g. protease inhibitors, siderophores) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[9] may be carried out

[9] Guidelines for oral toxicity studies have been developed in international fora, for example the *OECD Guidelines for the Testing of Chemicals* issued by the Organisation for Economic Co-operation and Development.

in cases where the protein is present in the food, but is not closely similar to proteins that have been safely consumed in food and has not previously been consumed safely in food, and taking into account its biological function in micro-organisms, where known.

- Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity, concentration and biological function of the substance and dietary exposure. The type of studies to be performed may include evaluations of metabolism, toxicokinetics, chronic toxicity/ carcinogenicity, impact on reproductive function, and teratogenicity.

41. The newly expressed or altered properties should be shown to be unrelated to any characteristics of donor organisms that could be harmful to human health. Information should be provided to ensure that genes coding for known toxins or antinutrients present in the donor organisms are not transferred to recombinant-DNA micro-organisms that do not normally express those toxic or antinutritious characteristics.

- Additional *in vivo* or *in vitro* studies may be needed on a case-by-case basis to assess the toxicity of expressed substances, taking into account the potential accumulation of any substances, toxic metabolites or antibiotics that might result from the genetic modification.

Compositional analyses of key components

42. Analyses of concentrations of key components[10] of foods produced by recombinant-DNA micro-organisms should be compared with an equivalent analysis of a conventional counterpart produced under the same conditions. The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. Ideally, the comparator(s) used in this assessment should be food produced using the near isogenic parent strain. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

Evaluation of metabolites

43. Some recombinant-DNA micro-organisms may be modified in a manner that could result in new or altered levels of various metabolites in foods produced using these organisms. Where altered metabolite levels are identified in foods, consideration should be given to the potential impacts on human health, using conventional procedures for

[10] Key nutrients or key antinutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major nutritional constituents (fats, proteins, carbohydrates), enzyme inhibitors as antinutrients, or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be produced by the micro-organism, such as those compounds whose toxic potency and level may be significant to health. Micro-organisms traditionally used in food processing are not usually known to produce such compounds under production conditions.

establishing the safety of such metabolites (e.g. procedures for assessing the human safety of chemicals in foods).

44. New or altered levels of metabolites produced by a recombinant-DNA micro-organism may change the population of micro-organisms in mixed culture, potentially increasing the risk for growth of harmful organisms or accumulation of harmful substances. Possible effects of genetic modification of a micro-organism on other micro-organisms should be assessed when a mixed culture of micro-organisms is used for food processing, such as for production of natural cheese, miso and soy sauce.

Effects of food processing

45. The potential effects of food processing, including home preparation, on foods produced using recombinant-DNA micro-organisms should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Therefore, information should be provided, describing the processing conditions used in the production of a food. For example, in the case of yoghurt, information should be provided on the growth of the organism and culture conditions.

Assessment of immunological effects

46. When the protein(s) resulting from an inserted gene is present in the food, it should be assessed for its potential to cause allergy. The likelihood that individuals may already be sensitive to the protein and whether a protein new to the food supply will induce allergic reactions should be considered. A detailed presentation of issues to be considered is presented in the Annex to this Guideline.

47. Genes derived from known allergenic sources should be assumed to encode an allergen and be avoided unless scientific evidence demonstrates otherwise. The transfer of genes from organisms known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

48. Recombinant-DNA micro-organisms that remain viable in foods may interact with the immune system in the gastrointestinal tract. Closer examination of these interactions will depend on the types of differences between the recombinant-DNA micro-organism and its conventional counterpart.

Assessment of viability and residence of micro-organisms in the human gastrointestinal tract

49. In some foods produced using recombinant-DNA micro-organisms, ingestion of these micro-organisms and their residence[11] may have an impact on the human intestinal

[11] Permanent life-long colonization by ingested micro-organisms is rare. Some orally administered micro-organisms have been recovered in faeces or in the colonic mucosa weeks after feeding ceased. Whether the genetically modified micro-organism is established in the gastrointestinal tract or not, the possibility remains that it might influence the microflora or the mammalian host (Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, *Safety assessment of foods derived from genetically modified micro-organism*, 24–28 September 2001, Geneva, Switzerland).

tract. The need for further testing of such micro-organisms should be based on the presence of their conventional counterpart in foods, and the nature of the intended and unintended effects of genetic modifications. If processing of the final food product eliminates viable micro-organisms (by heat treatment in baking bread, for example) or if accumulations of end-products toxic to the micro-organism (such as alcohol or acids) eliminate viability, then viability and residence of micro-organisms in the alimentary system need no examination.

50. For applications in which recombinant-DNA micro-organisms used in production remain viable in the final food product (e.g. organisms in some dairy products), it may be desirable to demonstrate the viability (or residence time) of the micro-organism alone and within the respective food matrix in the digestive tract, and the impact on the intestinal microflora in appropriate systems. The nature of intended and unintended effects of genetic modification and the degree of differences from the conventional counterpart will determine the extent of such testing.

Antibiotic resistance and gene transfer

51. In general, traditional strains of micro-organisms developed for food processing uses have not been assessed for antibiotic resistance. Many micro-organisms used in food production possess intrinsic resistance to specific antibiotics. Such properties need not exclude such strains from consideration as recipients in constructing recombinant-DNA micro-organisms. However, strains in which antibiotic resistance is encoded by transmissible genetic elements should not be used where such strains or these genetic elements are present in the final food. Any indication of the presence of plasmids, transposons and integrons containing such resistance genes should be specifically addressed.

52. Alternative technologies, demonstrated to be safe, that do not rely on antibiotic resistance marker genes in viable micro-organisms present in foods should be used for selection purposes in recombinant-DNA micro-organisms. In general, use of antibiotic resistance markers for constructing intermediate strains should pose no significant hazards that would exclude the use of the ultimate strains in food production, provided that the antibiotic resistance marker genes have been removed from the final construct.

53. Transfer of plasmids and genes between the resident intestinal microflora and ingested recombinant-DNA micro-organisms may occur. The possibility and consequences of gene transfer from recombinant-DNA micro-organisms and food products produced by recombinant-DNA micro-organisms to gut micro-organisms or human cells should also be considered. Transferred DNA would be unlikely to be maintained in the absence of selective pressure. Nevertheless, the possibility of such events cannot be completely discounted.

54. In order to minimize the possibility of gene transfer, the following steps should be considered:

A. Chromosomal integration of the inserted genetic material may be preferable to localization on a plasmid.
B. Where the recombinant-DNA micro-organism will remain viable in the gastrointestinal tract, genes that could provide a selective advantage to recipient organisms to which the genetic material is unintentionally transferred should be avoided in the genetic construct.
C. Sequences that mediate integration into other genomes should be avoided in constructing the introduced genetic material.

Nutritional modification

55. The assessment of possible compositional changes to key nutrients, which should be conducted for all foods produced using recombinant-DNA micro-organisms, has already been addressed under "Compositional analyses of key components". If such nutritional modifications have been implemented, the food should be subjected to additional testing to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

56. Information about the known patterns of use and consumption of a food and its derivatives should be used to estimate the likely intake of the food produced using the recombinant-DNA micro-organism. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups, such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.

57. The use of modern biotechnology to change nutrient levels in foods produced using micro-organisms could result in broad changes to the nutrient profile. The intended modification in the micro-organism could alter the overall nutrient profile of the product, which, in turn, could affect the nutritional status of individuals consuming the food. The impact of changes that could affect the overall nutrient profile should be determined.

58. When the modification results in a food product with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods whose nutritional composition is closer to that of the food produced using the recombinant-DNA micro-organism) as appropriate comparators to assess the nutritional impact of the food.

59. Some foods may require additional testing. For example, animal-feeding studies may be warranted for foods produced using recombinant-DNA micro-organisms if changes

in the bioavailability of nutrients are expected or if the composition is not comparable with conventional foods. In addition, foods designed for health benefits may require an assessment beyond the scope of this Guideline such as specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food.

Review of safety assessments

60. The goal of the safety assessment is a conclusion as to whether the food produced using a recombinant-DNA micro-organism is as safe as the conventional counterpart, taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

ANNEX

ASSESSMENT OF POSSIBLE ALLERGENICITY

SECTION 1 – INTRODUCTION

1. All newly expressed proteins[12] produced by recombinant-DNA micro-organisms that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein. Therefore, it is recommended that an integrated, stepwise, case-by-case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data as no single criterion is sufficiently predictive.

3. The end-point of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

SECTION 2 – ASSESSMENT STRATEGY

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including, but not limited to, its susceptibility to enzymatic degradation, heat stability and/or acid and enzymatic treatment.

5. As there is no single test that can predict the likely human immunoglobulin E (IgE) response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins produced by recombinant-DNA micro-organisms, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced by recombinant-DNA micro-organisms. Particular attention should be given to the choice of the expression host, as post-translational modifications allowed by different

[12] This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. The issue of enteropathies is addressed in paragraph 47 of the *Guideline for the conduct of food safety assessment of foods produced using recombinant-DNA micro-organisms* (CAC/GL 46-2003). In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

hosts (i.e. eukaryotic vs prokaryotic systems) may have an impact on the allergenic potential of the protein.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

SECTION 3 – INITIAL ASSESSMENT

Section 3.1 – Source of the protein

7. As part of the data supporting the safety of foods produced using recombinant-DNA micro-organisms, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

Section 3.2 – Amino acid sequence homology

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results.[13] Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35 percent identity in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically-based evaluation.

[13] It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segment searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives; inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also Sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

Section 3.3 – Pepsin resistance

12. Resistance to pepsin digestion has been observed in several food allergens; thus, a correlation exists between resistance to digestion by pepsin and allergenic potential.[14] Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided.[15]

SECTION 4 – SPECIFIC SERUM SCREENING

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals.[16] In addition, the

[14] The method outlined in *The United States Pharmacopoeia* (1995) was used in the establishment of the correlation (Astwood *et al.*, 1996).

[15] Reference to Joint FAO/WHO Expert Consultation (2001).

[16] According to the report of the Joint FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology (22–25 January 2001, Rome) a minimum of eight relevant sera is required in order to achieve a 99-percent certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols.[17] A positive result in such tests would indicate a potential allergen.

SECTION 5 – OTHER CONSIDERATIONS

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute towards an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing that would be applied and its effects on the presence of the protein in the final food product.

17. As scientific knowledge and technology evolve, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include: targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

[17] Reference to the joint FAO/WHO Expert Consultation (2001) on description of *ex vivo*.

GUIDELINE FOR THE CONDUCT OF FOOD SAFETY ASSESSMENT OF FOODS DERIVED FROM RECOMBINANT-DNA ANIMALS

CAC/GL 68-2008

SECTION 1 – SCOPE

1. This Guideline supports the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003). It addresses safety and nutritional aspects of foods consisting of, or derived from, animals that have a history of safe use as sources of food, and that have been modified by modern biotechnology to exhibit new or altered expression of traits.[1]

2. The development, raising and use of animals for human purposes and, in particular, for use for food raise a variety of issues beyond food safety. Without prejudice to their legitimacy or importance, or to whether or how the use of recombinant deoxyribonucleic acid (DNA) methods in developing animals for food use might affect those issues, this Guideline addresses only food safety and nutritional issues. Therefore, it does not address:
   - animal welfare;
   - ethical, moral and socio-economic aspects;
   - environmental risks related to the environmental release of recombinant-DNA animals used in food production;
   - the safety of recombinant-DNA animals used as feed, or the safety of animals fed with feed derived from recombinant-DNA animals, plants and micro-organisms.

3. The Codex Principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities, such as food additives and pesticide residues, or a specific chemical or microbial contaminant, that have identifiable hazards and risks; they are not intended to apply to whole foods as such. Indeed, few foods, whatever their origin, have been assessed scientifically in a manner that would fully characterize all risk associated with the food. Further, many foods contain substances that would probably be found harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. This approach is based on the principle that the safety of foods derived from new animal lines, including recombinant-DNA animals, is assessed relative to the conventional counterpart having a history of safe use, taking into account both intended and unintended effects. Rather than trying to identify every hazard associated with a

[1] This Guideline was developed primarily for animals bearing heritable recombinant-DNA constructs.

particular food, the intention is to identify new or altered hazards relative to the conventional counterpart.

5. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003). If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and, if necessary, further risk assessment, the food would be subjected to risk management considerations in accordance with the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003) before being considered for commercial distribution.

6. Risk management measures such as post-market monitoring of consumer health effects may assist the risk assessment process. These are discussed in paragraph 20 of the *Principles for the risk analysis of foods derived from modern biotechnology* (CAC/GL 44-2003).

7. The Guideline describes the recommended approach for the food safety assessment of foods derived from recombinant-DNA animals where a conventional counterpart exists, and identifies the data and information that are generally applicable to making such assessments.[2] In assessing the safety of food from recombinant-DNA animals, the approach should take into account all of the following:

   A. the nature of the recombinant-DNA construct and its expression product(s), if any;
   B. the health status of the recombinant-DNA animal; and
   C. the composition of foods produced from recombinant-DNA animals, including key nutrients.

   While this Guideline is designed for foods derived from recombinant-DNA animals, the approach described could, in general, be applied to foods derived from animals that have been altered by other techniques.[3]

8. A diverse range of animals is used as food or for food production (e.g. mammals, birds, finfish and shellfish) and may be modified using *in vitro* nucleic acid techniques. Because of the combined impacts of their genetic diversity, husbandry and conditions under which they are raised or harvested, assessment of food safety must be considered on a case-by-case basis, with due regard to the framework presented in this Guideline.

[2] The approach to the safety assessment of foods derived from recombinant-DNA animals was first discussed at the 1991 Joint FAO/WHO Consultation on strategies for assessing the safety of foods produced by biotechnology. Further elaboration of the recommended approach was undertaken at the 2003 Joint FAO/WHO Expert Consultation on the safety assessment of foods derived from genetically modified animals, including fish.

[3] The food safety assessment of foods derived from animals bearing non-heritable constructs may require additional specific consideration, e.g. regarding hazards identified in the 2007 Joint FAO/WHO Expert Consultation on the safety assessment of foods derived from recombinant-DNA animals.

SECTION 2 – DEFINITIONS

9. The definitions below apply to this Guideline:

Recombinant-DNA animal means an animal in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

Conventional counterpart means an animal breed with a known history of safe use as food from which the recombinant-DNA animal line was derived, as well as the breeding partners used in generating the animals ultimately used as food, and/or food derived from such animals.[4]

SECTION 3 – INTRODUCTION TO FOOD SAFETY ASSESSMENT

10. Traditionally, food products derived from animals developed through conventional breeding or obtained from wild species have not been systematically subjected to extensive chemical, toxicological or nutritional evaluation prior to marketing. Thus, although new breeds of animals are often evaluated by breeders for phenotypic characteristics, they are not subjected to the rigorous and extensive food safety testing procedures, including validated toxicity studies in test animals, that are typical of chemicals such as food additives or contaminants that may be present in food. Instead, food derived from an animal of known and acceptable health status has generally been considered suitable for human consumption.

11. The use of animal models for assessing toxicological end-points is a major element in the risk assessment of many compounds, such as pesticides. However, in most cases, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. Therefore, it is relatively straightforward to feed such compounds to test animals at a range of doses some several orders of magnitude greater than the expected human exposure levels in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

12. Studies using test animals cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds and often characterized by a wide variation in composition and nutritional value. Owing to their bulk and effect on satiety, they can usually only be fed to test animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects that are not related directly to the

[4] It is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can, therefore, be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed studies using test animals could be requested on the whole food. Another consideration in deciding the need for studies with test animals is whether it is appropriate to subject test animals to such a study if it is unlikely to give rise to meaningful information.

13. Owing to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, and based on the experience of assessing the safety of whole foods, a more focused approach is required for the safety assessment of food derived from animals, including recombinant-DNA animals. This has been addressed by the development of a multidisciplinary approach for assessing safety that takes into account both intended and unintended changes that may occur in the animal or in the food products derived from it, using the concept of substantial equivalence.

14. The concept of substantial equivalence is a key step in the safety assessment process. However, it is not a safety assessment in itself; rather, it represents the starting point that is used to structure the safety assessment of a new food relative to its conventional counterpart. This concept is used to identify similarities and differences between the new food and its conventional counterpart.[5] It aids in the identification of potential food safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods derived from recombinant-DNA animals. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the new product can be considered relative to its conventional counterpart.

Unintended effects

15. In achieving the objective of conferring a specific trait (intended effect) to an animal by the insertion of defined DNA sequences, additional traits could, in some cases, be acquired or existing traits could be lost or modified (unintended effects). The potential occurrence of unintended effects is not restricted to the use of *in vitro* nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in conventional breeding as well in association with the use of assisted reproductive technologies currently in use. Unintended effects may be deleterious, beneficial or neutral with respect to the health of the animal or the safety of the foods derived from the animal. Unintended effects in recombinant-DNA animal may also arise through the insertion of DNA sequences and/or they may arise through subsequent conventional breeding of the recombinant-DNA animal. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA animal would have an unexpected, adverse effect on human health.

[5] The concept of substantial equivalence as described in the report of the 2000 Joint FAO/WHO Expert Consultation (*Safety aspects of genetically modified foods of plant origin*, WHO/SDE/PHE/FOS/00.6, WHO, Geneva, 2000). The concept of substantial equivalence was further considered in the context of comparative safety assessment at the 2003 Joint FAO/WHO Expert Consultation on the Safety Assessment of Foods Derived from Genetically Modified Animals, including Fish.

16. Unintended effects can result from the random insertion of DNA sequences into the animal genome, which may cause disruption or silencing of existing genes, activation of silent genes, or modifications in the expression of existing genes. Unintended effects may also result in the formation of new or changed patterns of metabolites.

17. Unintended effects caused by *in vitro* nucleic acid techniques may be subdivided into two groups: those that are "predictable", and those that are "unexpected". Many unintended effects are largely predictable based on knowledge of the inserted trait and its metabolic connections or of the site of insertion. As knowledge of animal genomes grows and familiarity with *in vitro* nucleic acid techniques increases, it may become easier to predict unintended effects of a particular modification. For example, homologous recombination, where appropriate, allows precise gene placement and so may reduce the occurrence of unintended effects associated with random integration. Molecular biological and biochemical techniques can also be used to analyse changes that occur at the level of transcription and translation that could lead to unintended effects. These should all be considered on a case-by-case basis.

18. The safety assessment of food derived from recombinant-DNA animals involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information is necessary in order to assess unintended effects, because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, provide assurance that the food is unlikely to have an adverse effect on human health. The assessment of unintended effects takes into account the phenotypic characteristics of the animal that are typically monitored by breeders during animal production stock development and improvement. These assessments provide a first screen for recombinant-DNA animals exhibiting unintended traits. Recombinant-DNA animals that pass this screen are subjected to safety assessment as described in Sections 4 and 5.

Framework of food safety assessment

19. The safety assessment follows a stepwise process of addressing relevant factors that include:

A. general description of the recombinant-DNA animal;
B. description of the recipient animal prior to the modification[6] and its use as food or for food production;
C. description of the donor organism or other source(s) of the introduced recombinant-DNA;
D. description of the genetic modification(s) including the construct(s) used to introduce the recombinant-DNA;

[6] Not to be confused with a surrogate dam.

E. description of the methods used to produce the initial recombinant-DNA animal[7] and the processes to produce the recombinant-DNA animal ultimately used as food or for food production;
F. characterization of the genetic modification(s) in the recombinant-DNA animal ultimately used as food or for food production;
G. safety assessment:
   a) health status of the recombinant-DNA animal,
   b) expressed substances (non-nucleic acid substances),
   c) compositional analyses of key components,
   d) food storage and processing, and
   e) intended nutritional modification;
H. other considerations.

20. In certain cases, the characteristics of the food may necessitate additional data and information to address issues that are unique to the product under review.

21. Experiments intended to develop data for safety assessment should be designed and conducted in accordance with sound scientific concepts and principles as well as, where appropriate, good laboratory practice. Primary data should be made available to regulatory authorities upon request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. Analytical methods should be documented.[8]

22. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. Safety assessments should address the health aspects for the whole population, including immunocompromised individuals, infants, the elderly and individuals with food hypersensitivities. The expected end-point of such an assessment will be a conclusion regarding whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. Therefore, in essence, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed to protect the health of consumers and, if so, to make well-informed and appropriate decisions in this regard.

SECTION 4 – GENERAL CONSIDERATIONS

General description of the recombinant-DNA animal

23. A description of the recombinant-DNA animal being presented for safety assessment should be provided. This description should identify the introduced recombinant-DNA, the method by which the recombinant-DNA is introduced to the recipient animal and

[7] First animal produced as a result of introducing the recombinant-DNA construct. Sometimes referred to as the founder animal.

[8] Reference is made to the "General criteria for the selection of methods of analysis" in the *Codex Alimentarius Commission Procedural Manual*.

the recombinant-DNA animal ultimately used as food or for food production, as well as the purpose of the modification. The potential risk of introducing pathogenic elements (e.g. elements responsible for transmissible spongiform encephalopathies and other infectious disease) originating from biological materials used as sources or during the production should be considered. The description should be sufficient to aid in understanding the nature and types of food being submitted for safety assessment.

Description of the recipient animal prior to the modification and its use as food or for food production

24. A comprehensive description of the recipient animal prior to the modification should be provided. The necessary data and information should include, but need not be restricted to:
   A. common or usual name, scientific name, and taxonomic classification;
   B. history of development through breeding, in particular identifying traits that may adversely affect human health;
   C. information on the genotype and phenotype of the animal relevant to its safety, including any known toxicity or allergenicity, symbiosis with toxin-producing organisms, potential for colonization by human pathogens;
   D. information on the effect of feed, exercise and growth environment on food products; and
   E. history of safe use as food or for food production.

25. Relevant phenotypic information should be provided not only for the recipient animal prior to the modification, but also for related lines and for animals that have made or may make a significant contribution to the genetic background of the recipient animal prior to the modification, if applicable.

26. The history of use may include information on how the animals breed and grow, how their food products are obtained (e.g. harvest, slaughter, milking), and the conditions under which those food products are made available to the consumer (e.g. storage, transport, processing). The extent to which the food products provide important nutritional components to particular subgroups of the population, and what important macronutrients or micronutrients they contribute to the diet should also be considered.

Description of the donor organism or other source(s) of the introduced recombinant-DNA

27. Information should be provided:
   A. on whether the recombinant-DNA was synthesized and it is not from a known natural source;
   B. if derived from another organism:
      i) usual or common name of that organism;
      ii) scientific name;
      iii) taxonomic classification;
      iv) information about the natural history as concerns food safety;
      v) information on naturally occurring toxins, and allergens;

vi) for micro-organisms, additional information on pathogenicity (to humans or the animal) and the relationship to known human or animal pathogens;
vii) for donors of animal or viral origin, information on the source material (e.g. cell culture) that has been used, and its origins; and
viii) information on the past and present use, if any, in the food supply and exposure route(s) other than the intended food use (e.g. possible presence of contaminants).

It is particularly important to determine whether the recombinant-DNA sequences impart pathogenicity or toxin production, or have other traits that affect human health (e.g. allergenicity).

Description of the genetic modification(s) including the construct(s) used to introduce the recombinant-DNA

28. Sufficient information should be provided on the genetic modification to allow for the identification of all genetic material potentially delivered to the recipient animal and to provide the necessary information for the analysis of the data supporting the characterization of the DNA inserted into the recombinant-DNA animal ultimately used as food or for food production.

29. The description of the process of introducing and incorporating (if appropriate) the recombinant-DNA into the recipient animal should include:
A. information on the specific methodology used for the transformation;
B. information, if applicable, on the DNA used to modify the animal (e.g. genes coding for proteins used for packaging vectors), including the source, identity and expected function in the animal:
– if viral vectors or known zoonotic organisms have been used, information on their natural hosts, target organs, transmission mode, pathogenicity, and potential for recombination with endogenous or exogenous pathogens; and
C. intermediate host organisms including the organisms (e.g. bacteria) used to produce or process DNA for producing the initial recombinant DNA animal.

30. Information should be provided on the DNA to be introduced, including:
A. the primary DNA sequence if the recombinant-DNA was synthesized and it is not from a known natural source;
B. the characterization of all the genetic components including marker genes, regulatory and other elements affecting the expression and function of the DNA;
C. the size and identity;
D. the location and orientation of the sequence in the final vector/construct; and
E. the function.

Description of the methods used to produce the initial recombinant-DNA animal and the processes to produce the recombinant-DNA animal ultimately used as food or for food production

31. Information should be provided on the various techniques and processes that are used to introduce the recombinant-DNA to obtain the initial recombinant-DNA animal.

Examples of possible techniques may include transformation of gametes, microinjection of early embryos, nuclear transfer of transgenic cells.

32. A description of the methods used to demonstrate heritability should be provided, including descriptions of how heritability is attained (e.g. breeding mosaic animals to obtain true germ-cell transmissible insertions).

33. Although initial recombinant-DNA animals are generally not intended to be used as food or for food production, knowledge of the method to generate these animals may be useful in hazard identification.

34. Information should also be provided on how the initial recombinant-DNA animal leads to the production of the animal ultimately used as food or for food production. This information should, if applicable, include information on the breeding partners or surrogate dams, including genotype and phenotype, husbandry, and conditions under which they are raised or harvested.

35. The history of use of food products from the animals used to generate the animals ultimately used for food production from the initial recombinant-DNA animal (e.g. breeding partners, surrogate dams) may include information on how the animals breed and grow, how their food products are obtained (e.g. harvest, slaughter, milking), and the conditions under which those food products are made available to consumers (e.g. storage, transport, processing).

Characterization of the genetic modification(s) in the recombinant-DNA animal ultimately used as food or for food production

36. In order to provide clear understanding of the impact on the composition and safety of foods derived from recombinant-DNA animals, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out.

37. Information should be provided on the DNA insertions into the animal genome; this should include:

A. the characterization and description of the inserted genetic materials. This should include an analysis of the potential for mobilization or recombination of any construct material used;
B. the number of insertion sites;
C. the organization of the inserted genetic material at each insertion site including copy number and sequence data of the inserted material and of the surrounding region, sufficient to identify any substances expressed as a consequence of the inserted material, or, where scientifically more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food; and
D. identification of any open reading frames within the inserted DNA or created by insertion with contiguous animal genomic DNA, including those that could result in fusion proteins.

38. Information should be provided on any newly expressed substances in the recombinant-DNA animal; this should include:
   A. the gene product(s) (e.g. a protein or an untranslated ribonucleic acid [RNA]) or other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food;
   B. the function of the gene product(s);
   C. the phenotypic description of the new trait(s);
   D. the level and site of expression in the animal of the expressed gene product(s), and the levels of its metabolites in the food; and
   E. where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous messenger RNA (mRNA) or protein.

39. In addition, information should be provided to:
   A. demonstrate whether the arrangement of the genetic material used for insertion has been conserved or whether significant rearrangement have occurred upon integration;
   B. demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affected sites critical for its structure or function;
   C. demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are stable and are expressed as expected. It may be necessary to examine the inheritance of the DNA insert itself or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly;
   D. demonstrate whether the newly expressed trait(s) are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene;
   E. indicate whether there is any evidence to suggest that one gene (or several genes) in the recombinant-DNA animal has been affected by the transformation process; and
   F. confirm the identity and expression pattern of any new fusion proteins.

Safety assessment of the recombinant-DNA animal ultimately used as food or for food production

Health status of the recombinant-DNA animal

40. In contrast to the situation with plants, animals that have a history of safe use as sources of food generally do not contain genes encoding for toxic substances. Because of this, the health of a conventional animal has traditionally been used as a useful indicator of the safety of derived foods. The practice of only allowing animals with known and acceptable health status to enter the human food supply has been and continues to be an essential step in ensuring safe food.

41. An evaluation of the health of the animal is one of the essential steps in ensuring safety of food derived from recombinant-DNA animals. In undertaking this evaluation,

it is important to compare the health status of the recombinant-DNA animal with the health status of the appropriate conventional counterpart, taking into account developmental stage.

42. The evaluation should include the following:
A. general health and performance indicators, including behaviour, growth and development, general anatomy, and reproductive function, if appropriate;
B. physiological measures, including clinical and analytical parameters;
C. other species-specific considerations, where appropriate.

Expressed substances (non-nucleic acid substances)
Assessment of possible toxicity or bioactivity

43. *In vitro* nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in recombinant-DNA animals. The new substances can be conventional components of animal-derived foods, such as proteins, fats, carbohydrates and vitamins, that are novel in the context of that recombinant-DNA animal. New substances might also include new metabolites resulting from the activity of enzymes generated by the expression of introduced DNA.

44. It is recognized that the evaluation of the health status of the recombinant-DNA animals may give information about possible toxicity and bioactivity of the expressed substances. However, it is still generally expected that the safety assessment will include evaluation of these substances.

45. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible tissues and other derived food products of the recombinant-DNA animal, including variations and mean values. Current dietary exposure and possible effects on population subgroups should also be considered.

46. Information should be provided to ensure that genes coding for known toxins or antinutrients present in donor organisms, if applicable, are not transferred to recombinant-DNA animals that do not normally express those toxic or antinutritious characteristics. This assurance is particularly important in cases where food derived from the recombinant-DNA animal is processed differently from the donor organism, as conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate antinutrients or toxicants.

47. For the reasons described in Section 3, conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substances may be necessary.

48. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins as well as stability

to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[9] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, taking into account its biological function in the animal where known.

49. Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the animal of the substance and dietary exposure. The type of studies to be performed may include studies on metabolism, toxicokinetics, subchronic toxicity, chronic toxicity/carcinogenicity, reproduction and development toxicity according to the traditional toxicological approach.

50. In the case of newly expressed bioactive substances, recombinant-DNA animals should be evaluated for potential effects of those substances as part of the overall animal health evaluation. It is possible that such substances may be active in humans. Therefore, consideration should be given to potential dietary exposure to the substance, whether the substance is likely to be bioactive following consumption and, if so, its potential to exert effects in humans.

51. Assessment of potential toxicity may require the isolation of the new substance from the recombinant-DNA animal, or the synthesis or production of the substance from an alternative source, in which case, the material should be shown to be biochemically, structurally and functionally equivalent to that produced in the recombinant-DNA animal.

Assessment of possible allergenicity (proteins)

52. When the protein(s) resulting from the inserted gene is present in the food, it should be assessed for potential allergenicity in all cases. An integrated, stepwise, case-by-case approach used in the assessment of the potential allergenicity of the newly expressed protein(s) should rely upon various criteria used in combination (as no single criterion is sufficiently predictive on either allergenicity or non-allergenicity). As noted in paragraph 21, the data should be obtained using sound scientific methods. A detailed presentation of issues to be considered can be found in the Annex to this document.[10]

53. The transfer of genes from commonly allergenic foods should be avoided unless it is documented that the transferred gene does not code for an allergen.

[9] Guidelines for oral toxicity studies have been developed in international fora, for example, the *OECD Guidelines for the Testing of Chemicals* issued by the Organisation for Economic Co-operation and Development.

[10] The FAO/WHO Expert Consultation 2001 report, which includes reference to several decision trees, was used in developing the Annex to this Guideline.

Compositional analysis of key components

54. Analyses of concentrations of key components[11] of the recombinant-DNA animal and, especially those typical of the food, should be compared with an equivalent analysis of a conventional counterpart grown and bred under the same husbandry conditions. Depending on the species (and the nature of the modification), it may be necessary to make comparisons between products from recombinant-DNA animals and appropriate conventional counterparts raised under more than one set of typical husbandry conditions. The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. However, it should be acknowledged that, particularly in the case of certain animal species, the available number of samples may be limited and there is likely to be large variation between animals, even those bred and raised under the same husbandry conditions. The comparator(s) used in this assessment should ideally be matched in housing and husbandry conditions, breed, age, sex, parity, lactation, or laying cycle (where appropriate). In practice, this may not be feasible at all times, in which case, conventional counterparts as close as possible should be chosen. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that are nutritionally important or that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

Food storage and processing

55. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA animals should also be considered. For example, alterations could occur in the heat stability of a toxicant or the bioavailability of an important nutrient after processing. Therefore, information should be provided, describing the processing conditions used in the production of a food ingredient from the animal.

56. If the modification is intended to change storage or shelf-life, the impact of the modification on food safety and/or nutritional quality should be evaluated.

Intended nutritional modification

57. The assessment of possible compositional changes to key nutrients, which should be conducted for all recombinant-DNA animals, has already been addressed under "compositional analyses of key components". However, foods derived from recombinant-DNA animals that have undergone modification to alter nutritional quality or functionality intentionally should be subjected to additional nutritional assessment to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

[11] Key nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates as nutrients or enzyme inhibitors as antinutrients) or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the organism, such as those compounds whose toxic potency and level may be significant to health and allergens. In animals, the presence of toxicants would be rare, whereas the presence of allergens would be common in some species.

58. Information about the known patterns of use and consumption of a food and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA animal. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.

59. The use of animal breeding, including *in vitro* nucleic acid techniques, to change nutrient levels in animal-derived foods can result in broad changes to the nutrient profile in two ways. The intended modification in animal constituents could change the overall nutrient profile of the animal product, and this change could affect the nutritional status of individuals consuming the food. Unexpected alterations in nutrients could have the same effect. Although the recombinant-DNA animal components may be individually assessed as safe, the impact of the change on the overall nutrient profile should be determined.

60. When the modification results in a food product with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods or food components whose nutritional composition is closer to that of the food derived from the recombinant-DNA animal) as appropriate comparators to assess the nutritional impact of the food.

61. Because of geographical and cultural variation in food consumption patterns, nutritional changes to a specific food may have a greater impact in some geographical areas or in some cultural populations than in others. Some animal-derived foods serve as the major source of a particular nutrient in some populations. The nutrient and the populations affected should be identified.

62. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA animals if changes in the bioavailability of nutrients are expected or if the composition is not comparable with conventional foods. In addition, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.

SECTION 5 – OTHER CONSIDERATIONS

Potential altered accumulation or distribution of substances or micro-organisms significant to human health

63. Some recombinant-DNA animals may exhibit traits that may result in the potential for altered accumulation or distribution of xenobiotics (e.g. veterinary drug residues, metals), which may affect food safety. Similarly, the potential for altered colonization by and shedding of human pathogens or new symbiosis with toxin-producing organisms in the recombinant-DNA animal could have an effect on food safety. The safety assessment should take the potential for these alterations into account, and where such alterations are identified, consideration should be given to the potential impacts on human health using conventional procedures for establishing safety.

Use of antibiotic resistance marker genes

64. Alternative transformation technologies that do not result in antibiotic resistance marker genes in foods should be used in the future development of recombinant-DNA animals, where such technologies are available and demonstrated to be safe.

65. Gene transfer from animals and their food products to gut micro-organisms or human cells is considered a rare possibility because of the many complex and unlikely events that would need to occur consecutively. Nevertheless, the possibility of such events cannot be completely discounted.[12]

66. In assessing safety of foods containing antibiotic resistance marker genes, the following factors should be considered:

A. the clinical and veterinary use and importance of the antibiotic in question; (Certain antibiotics are the only drug available to treat some clinical conditions [e.g. vancomycin for use in treating certain staphylococcal infections]. Marker genes encoding resistance to such antibiotics should not be used in recombinant-DNA animals.)

B. whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficacy of orally administered antibiotic; and (This assessment should provide an estimate of the amount of orally ingested antibiotic that could be degraded by the presence of the enzyme in food, taking into account factors such as dosage of the antibiotic, amount of enzyme likely to remain in food following exposure to digestive conditions, including neutral or alkaline stomach conditions and the need for enzyme cofactors, e.g. adenosine triphosphate [ATP] for enzyme activity and estimated concentration of such factors in food.)

C. safety of the gene product, as would be the case for any other expressed gene product.

[12] In cases where there are high levels of naturally occurring bacteria that are resistant to the antibiotic, the likelihood of such bacteria transferring this resistance to other bacteria will be orders of magnitude higher than the likelihood of transfer between ingested foods and bacteria.

67. If evaluation of the data and information suggests that the presence of the antibiotic resistance marker gene or gene product presents risks to human health, the marker gene or gene product should not be present in foods. Antibiotic resistance genes used in food production that encode resistance to clinically used antibiotics should not be present in foods.

Review of safety assessments

68. The goal of the safety assessment is a conclusion as to whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

ANNEX

ASSESSMENT OF POSSIBLE ALLERGENICITY

SECTION 1 – INTRODUCTION

1. All newly expressed proteins[13] in recombinant-DNA animals that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein. Therefore, it is recommended that an integrated, stepwise, case-by-case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data as no single criterion is sufficiently predictive.

3. The end-point of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

SECTION 2 – ASSESSMENT STRATEGY

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including, but not limited to, its susceptibility to enzymatic degradation, heat stability and/or acid and enzymatic treatment.

5. As there is no single test that can predict the likely human immunoglobulin E (IgE) response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins from the recombinant-DNA animal, or the synthesis or production of the substance from an alternative source, in which case, the material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA animal. Particular attention should be given to the choice of the expression host, as post-translational modifications allowed by different hosts (i.e. eukaryotic vs prokaryotic systems) may have an impact on the allergenic potential of the protein.

[13] This assessment strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

SECTION 3 – INITIAL ASSESSMENT

Section 3.1 – Source of the protein

7. As part of the data supporting the safety of foods derived from recombinant-DNA animals, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

Section 3.2 – Amino acid sequence homology

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results.[14] Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35 percent identity in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to

[14] It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives; inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also Sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

Section 3.3 – Pepsin resistance

12. Resistance to pepsin digestion has been observed in several food allergens; thus, a correlation exists between resistance to digestion by pepsin and allergenic potential.[15] Therefore, the resistance of protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided.[16]

SECTION 4 – SPECIFIC SERUM SCREENING

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient number of individuals.[17] In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic and which do not

[15] The method outlined in *The United States Pharmacopoeia* (1995) was used in the establishment of the correlation (Astwood *et al.*, 1996).

[16] Report of the Joint FAO/WHO Expert Consultation on the allergenicity of foods derived from biotechnology (2001): *Evaluation of allergenicity of genetically modified foods*, Section 6.4 Pepsin resistance.

[17] According to the report of the Joint FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology (22–25 January 2001, Rome) a minimum of eight relevant sera is required in order to achieve a 99 percent certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols.[18] A positive result in such tests would indicate a potential allergen.

SECTION 5 – OTHER CONSIDERATIONS

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute towards an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing that would be applied and its effects on the presence of the protein in the final food product.

17. As scientific knowledge and technology evolve, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include: targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

[18] *Ex vivo* procedure is described as the testing for allergenicity using cells or tissue culture from allergic human subjects (report of Joint FAO/WHO Expert Consultation on allergenicity of foods derived from biotechnology).

523 pages withheld in their entirety

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Code of Federal Regulations

Title 21 - Food and Drugs

Volume: 2
Date: 2012-04-01
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Title: Section 110.5 - Current good manufacturing practice.
Context: Title 21 - Food and Drugs. CHAPTER I - FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN SERVICES (CONTINUED). SUBCHAPTER B - FOOD FOR HUMAN CONSUMPTION. PART 110 - CURRENT GOOD MANUFACTURING PRACTICE IN MANUFACTURING, PACKING, OR HOLDING HUMAN FOOD. Subpart A - General Provisions.

§ 110.5 Current good manufacturing practice.

(a) The criteria and definitions in this part shall apply in determining whether a food is adulterated (1) within the meaning of section 402(a)(3) of the act in that the food has been manufactured under such conditions that it is unfit for food; or (2) within the meaning of section 402(a)(4) of the act in that the food has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health. The criteria and definitions in this part also apply in determining whether a food is in violation of section 361 of the Public Health Service Act (42 U.S.C. 264).

(b) Food covered by specific current good manufacturing practice regulations also is subject to the requirements of those regulations.

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Title 21: Food and Drugs
PART 170—FOOD ADDITIVES
Subpart A—General Provisions

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§170.3 Definitions.

For the purposes of this subchapter, the following definitions apply:

(a) *Secretary* means the Secretary of Health and Human Services.

(b) *Department* means the Department of Health and Human Services.

(c) *Commissioner* means the Commissioner of Food and Drugs.

(d) As used in this part, the term *act* means the Federal Food, Drug, and Cosmetic Act approved June 25, 1936, 52 Stat. 1040 *et seq.*, as amended (21 U.S.C. 301-392).

(e)(1) *Food additives* includes all substances not exempted by section 201(s) of the act, the intended use of which results or may reasonably be expected to result, directly or indirectly, either in their becoming a component of food or otherwise affecting the characteristics of food. A material used in the production of containers and packages is subject to the definition if it may reasonably be expected to become a component, or to affect the characteristics, directly or indirectly, of food packed in the container. "Affecting the characteristics of food" does not include such physical effects, as protecting contents of packages, preserving shape, and preventing moisture loss. If there is no migration of a packaging component from the package to the food, it does not become a component of the food and thus is not a food additive. A substance that does not become a component of food, but that is used, for example, in preparing an ingredient of the food to give a different flavor, texture, or other characteristic in the food, may be a food additive.

(2) *Uses of food additives not requiring a listing regulation.* Use of a substance in a food contact article (e.g., food-packaging or food-processing equipment) whereby the substance migrates, or may reasonably be expected to migrate, into food at such levels that the use has been exempted from regulation as a food additive under §170.39, and food contact substances used in accordance with a notification submitted under section 409(h) of the act that is effective.

(3) *A food contact substance* is any substance that is intended for use as a component of materials used in manufacturing, packing, packaging, transporting, or holding food if such use is not intended to have any technical effect in such food.

(f) *Common use in food* means a substantial history of consumption of a substance for food use by a significant number of consumers.

(g) The word *substance* in the definition of the term "food additive" includes a food or food component consisting of one or more ingredients.

(h) *Scientific procedures* include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

(i) *Safe* or *safety* means that there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present

state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance. Safety may be determined by scientific procedures or by general recognition of safety. In determining safety, the following factors shall be considered:

(1) The probable consumption of the substance and of any substance formed in or on food because of its use.

(2) The cumulative effect of the substance in the diet, taking into account any chemically or pharmacologically related substance or substances in such diet.

(3) Safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food and food ingredients, are generally recognized as appropriate.

(j) The term *nonperishable processed food* means any processed food not subject to rapid decay or deterioration that would render it unfit for consumption. Examples are flour, sugar, cereals, packaged cookies, and crackers. Not included are hermetically sealed foods or manufactured dairy products and other processed foods requiring refrigeration.

(k) *General recognition of safety* shall be determined in accordance with §170.30.

(l) *Prior sanction* means an explicit approval granted with respect to use of a substance in food prior to September 6, 1958, by the Food and Drug Administration or the United States Department of Agriculture pursuant to the Federal Food, Drug, and Cosmetic Act, the Poultry Products Inspection Act, or the Meat Inspection Act.

(m) *Food* includes human food, substances migrating to food from food-contact articles, pet food, and animal feed.

(n) The following general food categories are established to group specific related foods together for the purpose of establishing tolerances or limitations for the use of direct human food ingredients. Individual food products will be included within these categories according to the detailed classifications lists contained in Exhibit 33B of the report of the National Academy of Sciences/National Research Council report, "A Comprehensive Survey of Industry on the Use of Food Chemicals Generally Recognized as Safe" (September 1972), which is incorporated by reference. Copies are available from the National Technical Information Service (NTIS), 5285 Port Royal Rd., Springfield, VA 22161, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.*

(1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving.

(2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix.

(3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks.

(4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals.

(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.

(6) Chewing gum, including all forms.

(7) Coffee and tea, including regular, decaffeinated, and instant types.

(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.

(9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars.

(10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products.

(11) Egg products, including liquid, frozen, or dried eggs, and egg dishes made therefrom, i.e., egg roll, egg foo young, egg salad, and frozen multicourse egg meals, but not fresh eggs.

(12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils.

(13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish.

(14) Fresh eggs, including cooked eggs and egg dishes made only from fresh shell eggs.

(15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals.

(16) Fresh fruits and fruit juices, including only raw fruits, citrus, melons, and berries, and home-prepared "ades" and punches made therefrom.

(17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom.

(18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made therefrom.

(19) Fresh vegetables, tomatoes, and potatoes, including only fresh and home-prepared vegetables.

(20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties.

(21) Fruit and water ices, including all frozen fruit and water ices.

(22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads.

(23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables.

(24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces.

(25) Hard candy and cough drops, including all hard type candies.

(26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors.

(27) Jams and jellies, home-prepared, including only home-prepared jams, jellies, fruit butters, preserves, and sweet spreads.

(28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads.

(29) Meat products, including all meats and meat containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation.

(30) Milk, whole and skim, including only whole, lowfat, and skim fluid milks.

(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products.

(32) Nuts and nut products, including whole or shelled tree nuts, peanuts, coconut, and nut and peanut spreads.

(33) Plant protein products, including the National Academy of Sciences/National Research Council "reconstituted vegetable protein" category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins.

(34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation.

(35) Processed fruits and fruit juices, including all commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, "ades", and drink substitutes made therefrom.

(36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends.

(37) Snack foods, including chips, pretzels, and other novelty snacks.

(38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies.

(39) Soups, home-prepared, including meat, fish, poultry, vegetable, and combination home-prepared soups.

(40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes.

(41) Sugar, white, granulated, including only white granulated sugar.

(42) Sugar substitutes, including granulated, liquid, and tablet sugar substitutes.

(43) Sweet sauces, toppings, and syrups, including chocolate, berry, fruit, corn syrup, and maple sweet sauces and toppings.

(o) The following terms describe the physical or technical functional effects for which direct human food ingredients may be added to foods. They are adopted from the National Academy of Sciences/National Research Council national survey of food industries, reported to the Food and Drug Administration under the contract title "A Comprehensive Survey of Industry on the Use of Food Chemicals Generally Recognized as Safe" (September 1972), which is incorporated by reference. Copies are available from the National Technical Information Service (NTIS), 5285 Port Royal Rd., Springfield, VA 22161, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.*

(1) *Anticaking agents and free-flow agents*: Substances added to finely powdered or crystalline food products to prevent caking, lumping, or agglomeration.

(2) *Antimicrobial agents*: Substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors, and the effects listed by the National Academy of Sciences/National Research Council under "preservatives."

(3) *Antioxidants*: Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation.

(4) *Colors and coloring adjuncts*: Substances used to impart, preserve, or enhance the color or shading of a food, including color stabilizers, color fixatives, color-retention agents, etc.

(5) *Curing and pickling agents*: Substances imparting a unique flavor and/or color to a food, usually producing an increase in shelf life stability.

(6) *Dough strengtheners*: Substances used to modify starch and gluten, thereby producing a more stable dough, including the applicable effects listed by the National Academy of Sciences/National Research Council under "dough conditioner."

(7) *Drying agents*: Substances with moisture-absorbing ability, used to maintain an environment of low moisture.

(8) *Emulsifiers and emulsifier salts*: Substances which modify surface tension in the component phase of an emulsion to establish a uniform dispersion or emulsion.

(9) *Enzymes*: Enzymes used to improve food processing and the quality of the finished food.

(10) *Firming agents*: Substances added to precipitate residual pectin, thus strengthening the supporting tissue and preventing its collapse during processing.

(11) *Flavor enhancers*: Substances added to supplement, enhance, or modify the original taste and/or aroma of a food, without imparting a characteristic taste or aroma of its own.

(12) *Flavoring agents and adjuvants*: Substances added to impart or help impart a taste or aroma in food.

(13) *Flour treating agents*: Substances added to milled flour, at the mill, to improve its color and/or baking qualities, including bleaching and maturing agents.

(14) *Formulation aids*: Substances used to promote or produce a desired physical state or texture in food, including carriers, binders, fillers, plasticizers, film-formers, and tableting aids, etc.

(15) *Fumigants*: Volatile substances used for controlling insects or pests.

(16) *Humectants*: Hygroscopic substances incorporated in food to promote retention of moisture, including moisture-retention agents and antidusting agents.

(17) *Leavening agents*: Substances used to produce or stimulate production of carbon dioxide in baked goods to impart a light texture, including yeast, yeast foods, and calcium salts listed by the National Academy of Sciences/National Research Council under "dough conditioners."

(18) *Lubricants and release agents*: Substances added to food contact surfaces to prevent ingredients and finished products from sticking to them.

(19) *Non-nutritive sweeteners*: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

(20) *Nutrient supplements*: Substances which are necessary for the body's nutritional and metabolic processes.

(21) *Nutritive sweeteners*: Substances having greater than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.

(22) *Oxidizing and reducing agents*: Substances which chemically oxidize or reduce another food ingredient, thereby producing a more stable product, including the applicable effect listed by the National Academy of Sciences/National Research Council under "dough conditioners."

(23) *pH control agents*: Substances added to change or maintain active acidity or basicity, including buffers, acids, alkalies, and neutralizing agents.

(24) *Processing aids*: Substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculents, filter aids, and crystallization inhibitors, etc.

(25) *Propellants, aerating agents, and gases*: Gases used to supply force to expel a product or used to reduce the amount of oxygen in contact with the food in packaging.

(26) *Sequestrants*: Substances which combine with polyvalent metal ions to form a soluble metal complex, to improve the quality and stability of products.

(27) *Solvents and vehicles*: Substances used to extract or dissolve another substance.

(28) *Stabilizers and thickeners*: Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.

(29) *Surface-active agents*: Substances used to modify surface properties of liquid food components for a variety of effects, other than emulsifiers, but including solubilizing agents, dispersants, detergents, wetting agents, rehydration enhancers, whipping agents, foaming agents, and defoaming agents, etc.

(30) *Surface-finishing agents*: Substances used to increase palatability, preserve gloss, and inhibit discoloration of foods, including glazes, polishes, waxes, and protective coatings.

(31) *Synergists*: Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.

(32) *Texturizers*: Substances which affect the appearance or feel of the food.

[42 FR 14483, Mar. 15, 1977, as amended at 47 FR 11835, Mar. 19, 1982; 53 FR 16546, May 10, 1988; 54 FR 24896, June 12, 1989; 60 FR 36595, July 17, 1995; 67 FR 35729, May 21, 2002]

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as a statistically significant difference with at least a probability (P) value of less than 0.05.

(4) The amount of the additive added for nutritive purposes plus the amount naturally present in free and combined (as protein) form does not exceed the following levels of amino acids expressed as percent by weight of the total protein of the finished food:

	Percent by weight of total protein (expressed as free amino acid)
L-Alanine	6.1
L-Arginine	6.6
L-Aspartic acid (including L-asparagine)	7.0
L-Cystine (including L-cysteine)	2.3
L-Glutamic acid (including L-glutamine)	12.4
Aminoacetic acid (glycine)	3.5
L-Histidine	2.4
L-Isoleucine	6.6
L-Leucine	8.6
L-Lysine	6.4
L- and DL-Methionine	3.1
L-Phenylalanine	5.8
L-Proline	4.2
L-Serine	8.4
L-Threonine	5.0
L-Tryptophan	1.8
L-Tyrosine	4.3
L-Valine	7.4

(d) Compliance with the limitations concerning PER under paragraph (c) of this section shall be determined by the method described in sections 43.212-43.218, "Official Methods of Analysis of the Association of Official Analytical Chemists," 13th Ed. (1980), which is incorporated by reference. Copies may be obtained from the AOAC INTERNATIONAL, 481 North Frederick Ave., suite 500, Gaithersburg, MD 20877, or may be examined at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html*. Each manufacturer or person employing the additive(s) under the provisions of this section shall keep and maintain throughout the period of his use of the additive(s) and for a minimum of 3 years thereafter, records of the tests required by this paragraph and other records required to assure effectiveness and compliance with this regulation and shall make such records available upon request at all reasonable hours by any officer or employee of the Food and Drug Administration, or any other officer or employee acting on behalf of the Secretary of Health and Human Services and shall permit such officer or employee to conduct such inventories of raw and finished materials on hand as he deems necessary and otherwise to check the correctness of such records.

(e) To assure safe use of the additive, the label and labeling of the additive and any premix thereof shall bear, in addition to the other information required by the Act, the following:

(1) The name of the amino acid(s) contained therein including the specific optical and chemical form.

(2) The amounts of each amino acid contained in any mixture.

(3) Adequate directions for use to provide a finished food meeting the limitations prescribed by paragraph (c) of this section.

(f) The food additive amino acids added as nutrients to special dietary foods that are intended for use solely under medical supervision to meet nutritional requirements in specific medical conditions and comply with the requirements of part 105 of this chapter are exempt from the limitations in paragraphs (c) and (d) of this section and may be used in such foods at levels not to exceed good manufacturing practices.

[42 FR 14491, Mar. 15, 1977; 42 FR 56728, Oct 28, 1977, as amended at 47 FR 11838, Mar. 19, 1982; 49 FR 10104, Mar. 19, 1984; 54 FR 24897, June 12, 1989; 59 FR 14550, Mar. 29, 1994; 61 FR 14480, Apr. 2, 1996]

§ 172.325 Bakers yeast protein.

Bakers yeast protein may be safely used in food in accordance with the following conditions:

(a) Bakers yeast protein is the insoluble proteinaceous material remaining after the mechanical rupture of yeast cells of *Saccharomyces cerevisiae* and removal of whole cell walls by centrifugation and separation of soluble cellular materials.

(b) The additive meets the following specifications on a dry weight basis:

(1) Zinc salts less than 500 parts per million (ppm) as zinc.

(2) Nucleic acid less than 2 percent.

(3) Less than 0.3 ppm arsenic, 0.1 ppm cadmium, 0.4 ppm lead, 0.05 ppm mercury, and 0.3 ppm selenium.

(c) The viable microbial content of the finished ingredient is:

(1) Less than 10,000 organisms/gram by aerobic plate count.

(2) Less than 10 yeasts and molds/gram.

(3) Negative for *Salmonella*, *E. coli*, coagulase positive *Staphylococci*, *Clostridium perfringens*, *Clostridium botulinum*, or any other recognized microbial pathogen or any harmful microbial toxin.

(d) The ingredient is used in food as a nutrient supplement as defined in §170.3(o)(20) of this chapter.

§ 172.330 Calcium pantothenate, calcium chloride double salt.

The food additive calcium chloride double salt of calcium pantothenate may be safely used in foods for special dietary uses in accordance with good manufacturing practice and under the following prescribed conditions:

(a) The food additive is of the *d* (dextrorotatory) or the *dl* (racemic) form.

(b) To assure safe use of the additive, the label and labeling of the food additive container, or that of any intermediate premixes prepared therefrom, shall bear, in addition to the other information required by the Act, the following:

(1) The name of the additive "calcium chloride double salt of *d*-calcium pantothenate" or "calcium chloride double salt of *dl*-calcium pantothenate", whichever is appropriate.

(2) A statement of the appropriate concentration of the additive, expressed as pantothenic acid.

§ 172.335 D-Pantothenamide.

The food additive D-pantothenamide as a source of pantothenic acid activity, may be safely used in foods for special dietary use in an amount not in excess of that reasonably required to produce its intended effect.

§ 172.340 Fish protein isolate.

(a) The food additive fish protein isolate may be safely used as a food supplement in accordance with the following prescribed conditions:

(1) The additive shall consist principally of dried fish protein prepared from the edible portions of fish after removal of the heads, fins, tails, bones, scales, viscera, and intestinal contents.

(2) The additive shall be derived only from species of bony fish that are generally recognized by qualified scientists as safe for human consumption and that can be processed as prescribed to meet the required specifications.

(3) Only wholesome fresh fish otherwise suitable for human consumption may be used. The fish shall be handled expeditiously under sanitary conditions. These conditions shall be in accordance with recognized good manufacturing practice for fish to be used as human food.

(4) The additive shall be prepared by extraction with hexane and food-grade ethanol to remove fat and moisture. Solvent residues shall be reduced by drying.

(b) The food additive meets the following specifications: (Where methods of determination are specified, they are Association of Official Analytical Chemists Methods, 13th ed., 1980, which are incorporated by reference).[1]

(1) Protein content, as N × 6.25, shall not be less than 90 percent by weight of the final product, as determined by the method described in section 2.057, Improved Kjeldahl Method for Nitrate-Free Samples (20)—Official Final Action.

(2) Moisture content shall not be more than 10 percent by weight of the final product, as determined by the method described in section 24.003, Air Drying (1)—Official First Action.

(3) Fat content shall not be more than 0.5 percent by weight of the final product, as determined by the method described in section 24.005, Crude Fat or Ether Extract—Official Final Action.

(4) Solvent residues in the final product shall not be more than 5 parts per

[1] Copies are available from: AOAC INTERNATIONAL, 481 North Frederick Ave., suite 500, Gaithersburg, MD 20877, or examined at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html*.

than 0.5 percent. The additive is added to the wort before or during cooking in the manufacture of beer.

§ 172.575 Quinine.

Quinine, as the hydrochloride salt or sulfate salt, may be safely used in food in accordance with the following conditions:

Uses	Limitations
In carbonated beverages as a flavor.	Not to exceed 83 parts per million, as quinine. Label shall bear a prominent declaration of the presence of quinine either by the use of the word "quinine" in the name of the article or through a separate declaration.

§ 172.580 Safrole-free extract of sassafras.

The food additive safrole-free extract of sassafras may be safely used in accordance with the following prescribed conditions:

(a) The additive is the aqueous extract obtained from the root bark of the plant *Sassafras albidum* (Nuttall) Nees (Fam. Lauraceae).

(b) It is obtained by extracting the bark with dilute alcohol, first concentrating the alcoholic solution by vacuum distillation, then diluting the concentrate with water and discarding the oily fraction.

(c) The purified aqueous extract is safrole-free.

(d) It is used as a flavoring in food.

§ 172.585 Sugar beet extract flavor base.

Sugar beet extract flavor base may be safely used in food in accordance with the provisions of this section.

(a) Sugar beet extract flavor base is the concentrated residue of soluble sugar beet extractives from which sugar and glutamic acid have been recovered, and which has been subjected to ion exchange to minimize the concentration of naturally occurring trace minerals.

(b) It is used as a flavor in food.

§ 172.590 Yeast-malt sprout extract.

Yeast-malt sprout extract, as described in this section, may be safely used in food in accordance with the following prescribed conditions:

(a) The additive is produced by partial hydrolysis of yeast extract (derived from *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, or *Candida utilis*) using the sprout portion of malt barley as the source of enzymes. The additive contains a maximum of 6 percent 5′ nucleotides by weight.

(b) The additive may be used as a flavor enhancer in food at a level not in excess of that reasonably required to produce the intended effect.

Subpart G—Gums, Chewing Gum Bases and Related Substances

§ 172.610 Arabinogalactan.

Arabinogalactan may be safely used in food in accordance with the following conditions:

(a) Arabinogalactan is a polysaccharide extracted by water from Western larch wood, having galactose units and arabinose units in the approximate ratio of six to one.

(b) It is used in the following foods in the minimum quantity required to produce its intended effect as an emulsifier, stabilizer, binder, or bodying agent: Essential oils, nonnutritive sweeteners, flavor bases, nonstandardized dressings, and pudding mixes.

§ 172.615 Chewing gum base.

The food additive chewing gum base may be safely used in the manufacture of chewing gum in accordance with the following prescribed conditions:

(a) The food additive consists of one or more of the following substances that meet the specifications and limitations prescribed in this paragraph, used in amounts not to exceed those required to produce the intended physical or other technical effect.

MASTICATORY SUBSTANCES

NATURAL (COAGULATED OR CONCENTRATED LATICES) OF VEGETABLE ORIGIN

Family	Genus and species
Sapotaceae:	
Chicle	Manilkara zapotilla Gilly and Manilkara chicle Gilly.
Chiquibul	Manilkara zapotilla Gilly.

	Limitations
Chlorine, as sodium hypochlorite, not to exceed 0.055 pound of chlorine per pound of dry starch; 0.45 percent of active oxygen obtained from hydrogen peroxide; and propylene oxide, not to exceed 25 percent. Sodium hydroxide, not to exceed 1 percent.	Residual propylene chlorohydrin not more than 5 parts per million in food starch-modified.

(h) Food starch may be modified by a combination of the treatments prescribed by paragraphs (a), (b), and/or (i) of this section and any one of the treatments prescribed by paragraph (c), (d), (e), (f), or (g) of this section, subject to any limitations prescribed by the paragraphs named.

(i) Food starch may be modified by treatment with the following enzymes:

Enzyme	Limitations
Alpha-amylase (E.C. 3.2.1.1) ..... Beta-amylase (E.C. 3.2.1.2). Glucoamylase (E.C. 3.2.1.3). Isoamylase (E.C. 3.2.1.68). Pullulanase (E.C. 3.2.1.41).	The enzyme must be generally recognized as safe or approved as a food additive for this purpose. The resulting nonsweet nutritive saccharide polymer has a dextrose equivalent of less than 20.

[42 FR 14491, Mar. 15, 1977, as amended at 43 FR 11697, Mar. 21, 1978; 48 FR 32015, June 19, 1981; 57 FR 54700, Nov. 20, 1992; 58 FR 21100, Apr. 19, 1993; 66 FR 17509, Apr. 2, 2001]

§ 172.894 Modified cottonseed products intended for human consumption.

The food additive modified cottonseed products may be used for human consumption in accordance with the following prescribed conditions:

(a) The additive is derived from:

(1) Decorticated, partially defatted, cooked, ground cottonseed kernels; or

(2) Decorticated, ground cottonseed kernels, in a process that utilizes *n*-hexane as an extracting solvent in such a way that no more than 60 parts per million of *n*-hexane residues and less than 1 percent fat by weight remain in the finished product; or

(3) Glandless cottonseed kernels roasted to attain a temperature of not less than 250 °F in the kernel for not less than 5 minutes for use as a snack food, or in baked goods, or in soft candy; or

(4) Raw glandless cottonseed kernels may be used in hard candy where the kernel temperature during cooking will exceed 250 °F for not less than 5 minutes.

(b) The additive is prepared to meet the following specifications:

(1) Free gossypol content not to exceed 450 parts per million.

(2) It contains no added arsenic compound and therefore may not exceed a maximum natural background level of 0.2 part per million total arsenic, calculated as As.

(c) To assure safe use of the additive, the label of the food additive container shall bear, in addition to other information required by the act, the name of the additive as follows:

(1) The additive identified in paragraph (a)(1) of this section as "partially defatted, cooked cottonseed flour".

(2) The additive identified in paragraph (a)(2) of this section as "defatted cottonseed flour".

(3) The additive identified in paragraph (a)(3) of this section as "roasted glandless cottonseed kernels".

(4) The additive identified in paragraph (a)(4) of this section as "raw glandless cottonseed kernels for use in cooked hard candy".

(d) The Food and Drug Administration and the Environmental Protection Agency have determined that glandless cottonseed kernels permitted for use by this section are a distinct commodity from glanded cottonseed.

§ 172.896 Dried yeasts.

Dried yeast (*Saccharomyces cerevisiae* and *Saccharomyces fragilis*) and dried torula yeast (*Candida utilis*) may be safely used in food provided the total folic acid content of the yeast does not exceed 0.04 milligram per gram of yeast (approximately 0.008 milligram of pteroylglutamic acid per gram of yeast).

§ 172.898 Bakers yeast glycan.

Bakers yeast glycan may be safely used in food in accordance with the following conditions:

(a) Bakers yeast glycan is the comminuted, washed, pasteurized, and

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2013]
[CITE: 21CFR172.898]

TITLE 21--FOOD AND DRUGS
CHAPTER I--FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH AND HUMAN SERVICES
SUBCHAPTER B--FOOD FOR HUMAN CONSUMPTION (CONTINUED)

PART 172 -- FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION

Subpart I--Multipurpose Additives

Sec. 172.898 Bakers yeast glycan.

Bakers yeast glycan may be safely used in food in accordance with the following conditions:

(a) Bakers yeast glycan is the comminuted, washed, pasteurized, and dried cell walls of the yeast, *Saccharomyces cerevisiae*. It is composed principally of long chain carbohydrates, not less than 85 percent on a dry solids basis. The carbohydrate is composed of glycan and mannan units in approximately a 2:1 ratio.

(b) The additive meets the following specifications on a dry weight basis: Less than 0.4 part per million (ppm) arsenic, 0.13 ppm cadmium, 0.2 ppm lead, 0.05 ppm mercury, 0.09 ppm selenium, and 10 ppm zinc.

(c) The viable microbial content of the finished ingredient is:

(1) Less than 10,000 organisms/gram by aerobic plate count.

(2) Less than 10 yeasts and molds/gram.

(3) Negative for *Salmonella*, *E. coli*, coagulase positive *Staphylococci*, *Clostridium perfringens*, *Clostridium botulinum*, or any other recognized microbial pathogen or any harmful microbial toxin.

(d) The additive is used or intended for use in the following foods when standards of identity established under section 401 of the Act do not preclude such use:

Use	Limitations
(1) In salad dressings as an emulsifier and emulsifier salt as defined in 170.3(o)(8) of this chapter, stabilizer and thickener as defined in 170.3(o)(28) of this chapter, or texturizer as defined in 170.3(o)(32) of this chapter	Not to exceed a concentration of 5 percent of the finished salad dressing.
(2) In frozen dessert analogs as a stabilizer and thickener as defined in 170.3(o)(28) of this chapter, or texturizer as defined in 170.3(o)(32) of this chapter	In an amount not to exceed good manufacturing practice.
(3) In sour cream analogs as a stabilizer and thickener as defined in 170.3(o)(28) of this chapter, or texturizer as defined in 170.3(o)(32) of this chapter	Do.
(4) In cheese spread analogs as a stabilizer and thickener as defined in 170.3(o)(28) of this chapter, or texturizer as defined in 170.3(o)(32) of this chapter	Do.
(5) In cheese-flavored and sour cream-flavored snack dips as a stabilizer and thickener as defined in 170.3(o)(28) of this chapter, or texturizer as	Do.

defined in 170.3(o)(32) of this chapter

(e) The label and labeling of the ingredient shall bear adequate directions to assure that use of the ingredient complies with this regulation.

[42 FR 14491, Mar. 15, 1977, as amended at 45 FR 58836, Sept. 5, 1980]

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prescribed in section 16.057 (liquid sample), entitled "Gravimetric Method—Official Final Action" under the heading "Lactose," or in section 31.061 (dry sample), entitled "Lane-Eynon General Volumetric Method" under the heading "Lactose—Chemical Methods—Official Final Action."

(v) Moisture content, 1 to 6 percent—as determined by the methods prescribed in section 16.192, entitled "Moisture (41)—Official Final Action" under the heading "Dried Milk, Nonfat Dry Milk, and Malted Milk."

(vi) Solids content, variable—as determined by the methods prescribed in section 16.032, entitled "Method I—Official Final Action" under the heading "Total Solids."

(vii) Titratable Acidity, variable—as determined by the methods prescribed in section 16.023, entitled "Acidity (2)—Official Final Action" under the heading "Milk," or by an equivalent potentiometric method.

(2) Limits of impurities are: Heavy metals (as lead). Not more than 10 parts per million (0.001 percent), as determined by the method described in the "Food Chemicals Codex," 4th ed. (1996), pp. 760–761, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, Box 285, 2101 Constitution Ave. NW., Washington, DC 20055 (Internet address *http://www.nap.edu*), or may be examined at the Center for Food Safety and Applied Nutrition's Library, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202–741–6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.*

(3) The whey protein concentrate shall be derived from milk that has been pasteurized, or the whey protein concentrate shall be subjected to pasteurization techniques or its equivalent before use in food.

(c) The whey protein concentrate may be used in food in accordance with good manufacturing practice as indicated in § 184.1(b)(1).

(d) The percent of protein present on a dry product basis, i.e., "whey protein concentrate (___% protein)," shall be declared on the label of the package sold to food manufacturers. The percent of protein may be declared in 5-percent increments, expressed as a multiple of 5, not greater than the actual percentage of protein in the product, or as an actual percentage provided that an analysis of the product on which the actual percentage is based is supplied to the food manufacturer.

(e) The presence of whey protein concentrate in a finished food product shall be listed as "whey protein concentrate".

[46 FR 44441, Sept. 4, 1981, as amended at 54 FR 24899, June 12, 1989; 64 FR 1761, Jan. 12, 1999]

§ 184.1983 Bakers yeast extract.

(a) Bakers yeast extract is the food ingredient resulting from concentration of the solubles of mechanically ruptured cells of a selected strain of yeast, *Saccharomyces cerevisiae*. It may be concentrated or dried.

(b) The ingredient meets the following specifications on a dry weight basis: Less than 0.4 part per million (ppm) arsenic, 0.13 ppm cadmium, 0.2 ppm lead, 0.05 ppm mercury, 0.09 ppm selenium, and 10 ppm zinc.

(c) The viable microbial content of the finished ingredient as a concentrate or dry material is:

(1) Less than 10,000 organisms/gram by aerobic plate count.

(2) Less than 10 yeasts and molds/gram.

(3) Negative for *Salmonella*, *E. coli*, coagulase positive *Staphylococci*, *Clostridium perfringens*, *Clostridium botulinum*, or any other recognized microbial pathogen or any harmful microbial toxin.

(d) The ingredient is used as a flavoring agent and adjuvant as defined in § 170.3(o)(12) of this chapter at a level not to exceed 5 percent in food.

(e) This regulation is issued prior to general evaluation of use of this ingredient in order to affirm as GRAS the specific use named.

Basic Report

Nutrient data for 18375, Leavening agents, yeast, baker's, active dry

Report Date:08-29-2013T09:32

Nutrient values and weights are for edible portion

Nutrient	Unit	1 Value Per100 g	1.0 tsp 4g	1.0 tbsp 12g	1.0 packet 7.2g
Proximates					
Water	g	5.08	0.20	0.61	0.37
Energy	kcal	325	13	39	23
Protein	g	40.44	1.62	4.85	2.91
Total lipid (fat)	g	7.61	0.30	0.91	0.55
Carbohydrate, by difference	g	41.22	1.65	4.95	2.97
Fiber, total dietary	g	26.9	1.1	3.2	1.9
Sugars, total	g	0.00	0.00	0.00	0.00
Minerals					
Calcium, Ca	mg	30	1	4	2
Iron, Fe	mg	2.17	0.09	0.26	0.16
Magnesium, Mg	mg	54	2	6	4
Phosphorus, P	mg	637	25	76	46
Potassium, K	mg	955	38	115	69
Sodium, Na	mg	51	2	6	4
Zinc, Zn	mg	7.94	0.32	0.95	0.57
Vitamins					
Vitamin C, total ascorbic acid	mg	0.3	0.0	0.0	0.0
Thiamin	mg	10.990	0.440	1.319	0.791
Riboflavin	mg	4.000	0.160	0.480	0.288
Niacin	mg	40.200	1.608	4.824	2.894
Vitamin B-6	mg	1.500	0.060	0.180	0.108
Folate, DFE	µg	2340	94	281	168
Vitamin B-12	µg	0.07	0.00	0.01	0.01
Vitamin A, RAE	µg	0	0	0	0
Vitamin A, IU	IU	0	0	0	0
Vitamin E (alpha-tocopherol)	mg	0.00	0.00	0.00	0.00
Vitamin D (D2 + D3)	µg	0.0	0.0	0.0	0.0
Vitamin D	IU	0	0	0	0
Vitamin K (phylloquinone)	µg	0.4	0.0	0.0	0.0
Lipids					
Fatty acids, total saturated	g	1.001	0.040	0.120	0.072
Fatty acids, total monounsaturated	g	4.309	0.172	0.517	0.310
Fatty acids, total polyunsaturated	g	0.017	0.001	0.002	0.001
Cholesterol	mg	0	0	0	0

Basic Report

Nutrient data for 18375, Leavening agents, yeast, baker's, active dry

Report Date:08-29-2013T09:32

Nutrient values and weights are for edible portion

Nutrient	Unit	1 Value Per100 g	1.0 tsp 4g	1.0 tbsp 12g	1.0 packet 7.2g
Other					
Caffeine	mg	0	0	0	0

27 pages withheld in their entirety

Pursuant FOIA exemption (b)(4) and

in accordance with copyright laws

Trull, Chelsea T-2

Subject: FW: AGRN-20 - DSM GE Yeast - requested revision
Attachments: GRAS dossier AGRN-20 - Revised Oct2016.pdf

From: La-Marta, James [mailto:James.LaMarta@dsm.com]
Sent: Monday, October 31, 2016 4:19 PM
To: Wong, Geoffrey K; Hendricks, Thomas T
Subject: AGRN-20 - DSM GE Yeast - requested revision

Dear Mr. Wong and Dr. Hendricks,

Per our conversation of Friday, 28 October I have revised the intended use statement to be in concert with the animals listed in the executive summary.
I have also clarified that the substrate for the fermentation is grain or grain by-products; removing the word (b)(4).
The attached file is only the main body of the Notice since you already have the annexes and references.

Kind Regards,

Jim La Marta, Ph.D.

James La Marta | Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Boulevard | Parsippany , NJ 07054 - 1298 | United States of America | T: 1-973-257-8347 | F: 1-973-257-8414 | M: (b)(6) |

THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED

By
Bio-based Products & Services
DSM Innovation Company

Melina Rumelhard,
Delft, The Netherlands

James La Marta,
Parsippany, NJ

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1. General introduction and claim of exemption from premarket approval requirements

1.1 Executive Summary

The purpose of this document is to provide technical and scientific information that supports the Generally Recognized as Safe (GRAS) status of DSM's Inactivated Modified *Saccharomyces cerevisiae* (also marketed as Advanced *Saccharomyces cerevisiae*) as a nutritional product for use in poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets, based on scientific procedures.

DSM Bio-based Products and Services (BPS) developed the yeast; our sister company, DSM Nutritional Products will manufacture and package the product. DSM-BPS will market the product. Inactivated Modified *Saccharomyces cerevisiae* is used by bio-ethanol facilities for the fermentation of sugars released from plant-based feed stocks, such as corn, wheat and sorghum into ethanol in a manner similar to the production of beer or grain neutral spirits used for in food and pharmaceutical processing. The yeast is not meant for fermentation of cellulosic feedstock.

Although the yeast was developed for use in bio-ethanol production from food grade substrates such as corn, wheat, sorghum and grain by-products, DSM-BPS is aware that potential customers may separate the inactivated yeast from the spent stillage for sale as an ingredient for animal feed or it will be dried with the cellulosic material in the stillage and become part of the Wet or Dried Distillers Grains with or without solubles (DDG/S and DDG) depending upon the business practices of the potential customer. These practices are commonly followed today with conventional *Saccharomyces cerevisiae* and the use of the yeast and DDG/S are permitted under feed ingredient definitions of the American Association of Feed Control Officers (AAFCO) as overseen by FDA's Center for Veterinary Medicine, at levels not exceeding good manufacturing procedures (AAFCO O.P. Sections 96 and 27 respectively). DSM-BPS does not intend to exceed the current limits for use of yeast and merely seeks to expand its permitted use to a genetically engineered strain.

The subject yeast, used for bio-ethanol production, will be produced in two product forms: a concentrated 'cream yeast' and a dry form commonly referred to as 'active dry yeast'. It is anticipated that the dry form will be the predominant marketed form for bio-ethanol production. Initially the Modified *Saccharomyces cerevisiae* is produced by (b)(4) in a facility that follows food cGMP (21 CFR 110). The yeast is then used in bio-ethanol production where it multiplies several times during the fermentation cycle. Since the host organism is safe and the incorporated DNA does not encode any known harmful or toxic substances, the resulting genetically engineered organism is considered to be safe for use in bio-ethanol production, and the resulting by-products containing the inactivated yeast are also considered safe.

Saccharomyces cerevisiae is a ubiquitous microorganism found all over the globe and has been utilized by humans serendipitously and intentionally for the production of bread, beer and wine for over six thousand years. The National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules (Department of Health and Human Services 1986) considers *Saccharomyces cerevisiae* a safe organism.

The safety of the Inactivated Modified *Saccharomyces cerevisiae* has been established according to the published literature. A comprehensive search of the scientific literature for safety and toxicity information on *Saccharomyces cerevisiae* was conducted by DSM Bio-based Products

and Services. The databases searched included Medline, BIOSIS and EMBASE databases as provided by the host STN.

The compositional equivalence of Modified *Saccharomyces cerevisiae* is shown by the nutritional analysis of two pilot plant batches and a sub-sample of one commercial batch of Modified *Saccharomyces cerevisiae* as compared to the published nutritional data of active dry baker's yeast and to the nutritional analysis of a sample of commercial baker's yeast. As anticipated, because the Modified *Saccharomyces cerevisiae* is the same in basic molecular composition as conventional yeast there was no significant difference between the different yeast products. Additionally, a study conducted to ascertain if excessive amounts of sugar alcohols or organic acids were produced due to the altered metabolism of the yeast did not reveal any differences compared to the wild type parent or a commercially available yeast used for bioethanol manufacture. Therefore we believe the Modified *Saccharomyces cerevisiae* is substantially similar to traditional yeast utilized in animal food.

All relevant information was reviewed, summarized and incorporated into a GRAS dossier, 'THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED' and submitted to the GRAS Panel. The GRAS Panel received information pertaining to the method of manufacture, product specification, analytical data, intended use levels in animal food for avian, ruminant and porcine species, resulting consumption estimates from the intended uses, and any other relevant data on safety and tolerance-related information. The members of the Expert Panel were Mark Cook, PhD, John A. Thomas, PhD, Fellow, ATS, and Stanley M. Tarka, Jr., PhD (Panel Chair).

1.2 Administrative information

1.2.1 Name and address of Notifier

DSM Innovation, Inc.
BioProducts & Services Division
45 Waterview Blvd.
Parsippany, New Jersey, 07054, USA
Tel: 973-257-8500

Person responsible for the dossier:

James La Marta, Ph.D., CFS
45 Waterview Boulevard
Parsippany, New Jersey 07054
Tel: 973-257-8325

1.2.2 Name and Address of Manufacturer

DSM Nutritional Products
1416 Willamsburg County Hwy
Kingstree, NC 29556

1.3 Name and Address of the Distributor

DSM Innovation, Inc.
BioProducts & Services Division
45 Waterview Blvd
Parsippany, NJ 07054

1.4 Common or usual name of the notified substance

The substance that is the subject of this GRAS notification is:

Inactivated Modified *Saccharomyces cerevisiae*

It will be sold alone or as a portion of Distillers Dried Grains with or without Solubles (DDG /DDGS).

1.5 Intended conditions of use and technical effects of the notified substance

The Inactivated Modified *Saccharomyces cerevisiae* will be used as a nutrient source for production animals; poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets. It will be present in dry distillers' grains with or without solubles at up to 20% of the dry solids.

1.6 Basis for GRAS determination

Pursuant to 21 C.F.R. § 570.30(a)(1), a panel of independent experts assembled by DSM, the GRAS Panel, has been asked to review, through scientific procedures, to determine if Inactivated Modified *Saccharomyces cerevisiae* that could be sold as a single feed ingredient or become part of Distillers Dried Grains with or without Solubles and is intended to be used as a nutrient source, is substantially equivalent to the host strain, common baker's yeast, from which it is derived, and is therefore Generally Recognized As Safe for use as an ingredient in animal food.

1.7 GRAS Exemption Claim

DSM Food Specialties provided the appropriate information on the safety and utility of the notified substance to an independent panel of qualified experts, the GRAS Panel for their evaluation. The enclosed dossier contains the information on the identity of the production organism, manufacture of the commercial product and information supporting the safety of its intended use. Also included are copies of the pertinent literature and other supportive data.

(b)(4) meeting appropriate food-grade specifications as described in this dossier and manufactured consistent with current Good Manufacturing Practices (cGMP), is Generally Recognized As Safe (GRAS) based on scientific procedures and is therefore exempt from the requirement for premarket approval noted in Section 201 (s) of the Federal Food Drug and Cosmetic Act.

1.8 Availability of information for FDA review

The data and information that are the basis for DSM's GRAS determination are available for the FDA's review and copying upon request during normal business hours at:

DSM Innovation, Inc.

BioProducts & Services Division

45 Waterview Blvd

Parsippany, NJ 07054

Tel: 973-257-8500

James La Marta, Ph.D., CFS

Date: 25 March 2016

2. Detailed information about the identity and manufacture of the notified substance CONFIDENTIAL

The subject of this notice is the yeast, Modified *Saccharomyces cerevisiae*' inactivated by the biofuel manufacturing process.

2.1 Description of Modified *Saccharomyces cerevisiae*

Table 2-1 Description of Mofdified *Saccharomyces cerevisiae*

Generic name	*Saccharomyces cerevisiae*
Synonyms	Baker's yeast, Brewer's Yeast
Chemical abstract service number (CAS)	(b)(4)

2.1.1 Taxonomy

Kingdom: Fungi
Phylum: Ascomycota
Class: Saccharomycetes
Order: Saccharomycetales
Family: Saccharomycetaceae
Genus: *Saccharomyces*
Species: *cerevisiae*

The Modified *Saccharomyces cerevisiae* is taxonomically identical to traditional yeast that is used in animal food.

2.2 Modifications introduced to the microorganism

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Nevertheless, the primary products produced by fermentations utilizing the engineered yeast are yeast biomass and ethanol and in that respect our engineered yeast is not different from the conventional yeast normally used for alcoholic fermentations.

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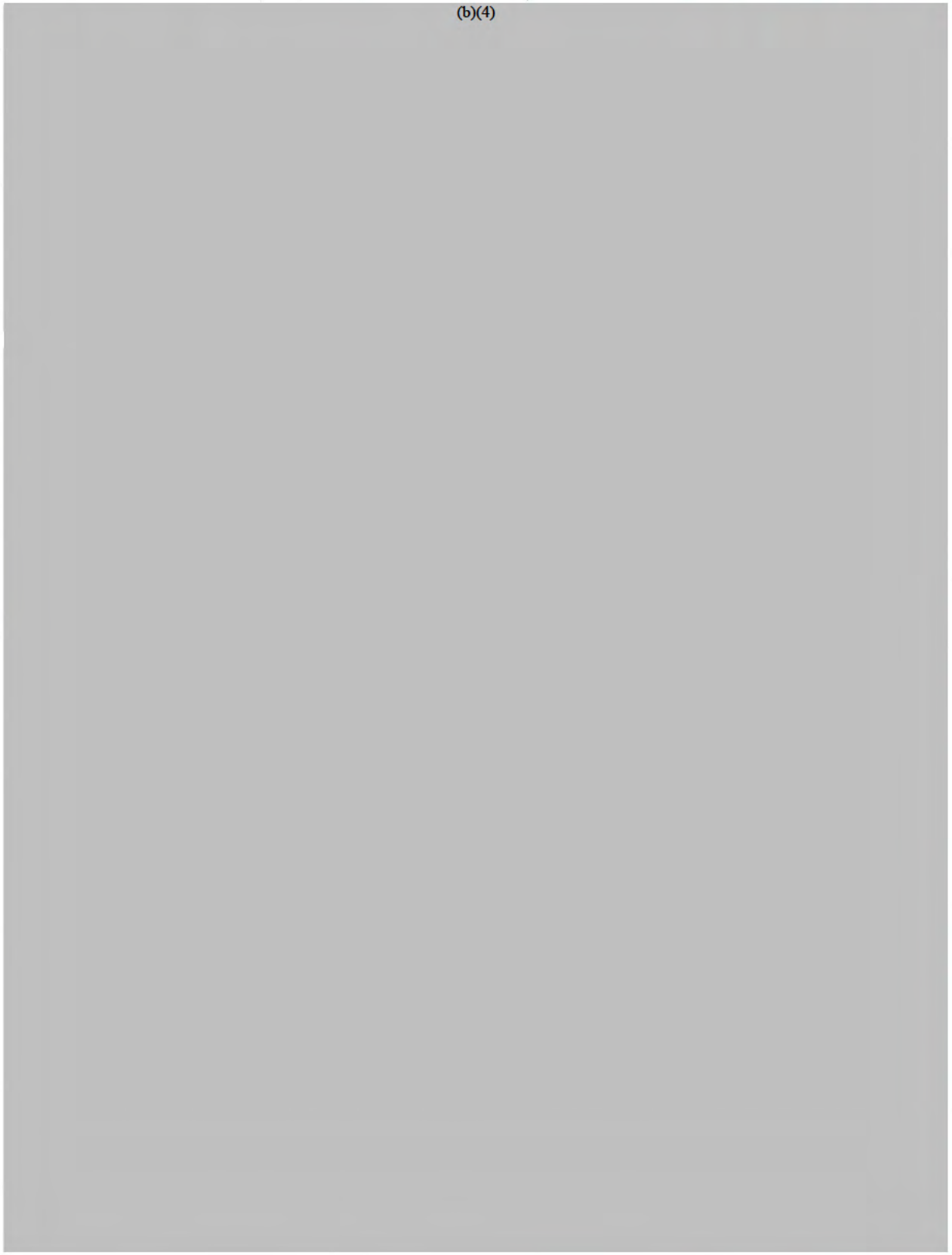

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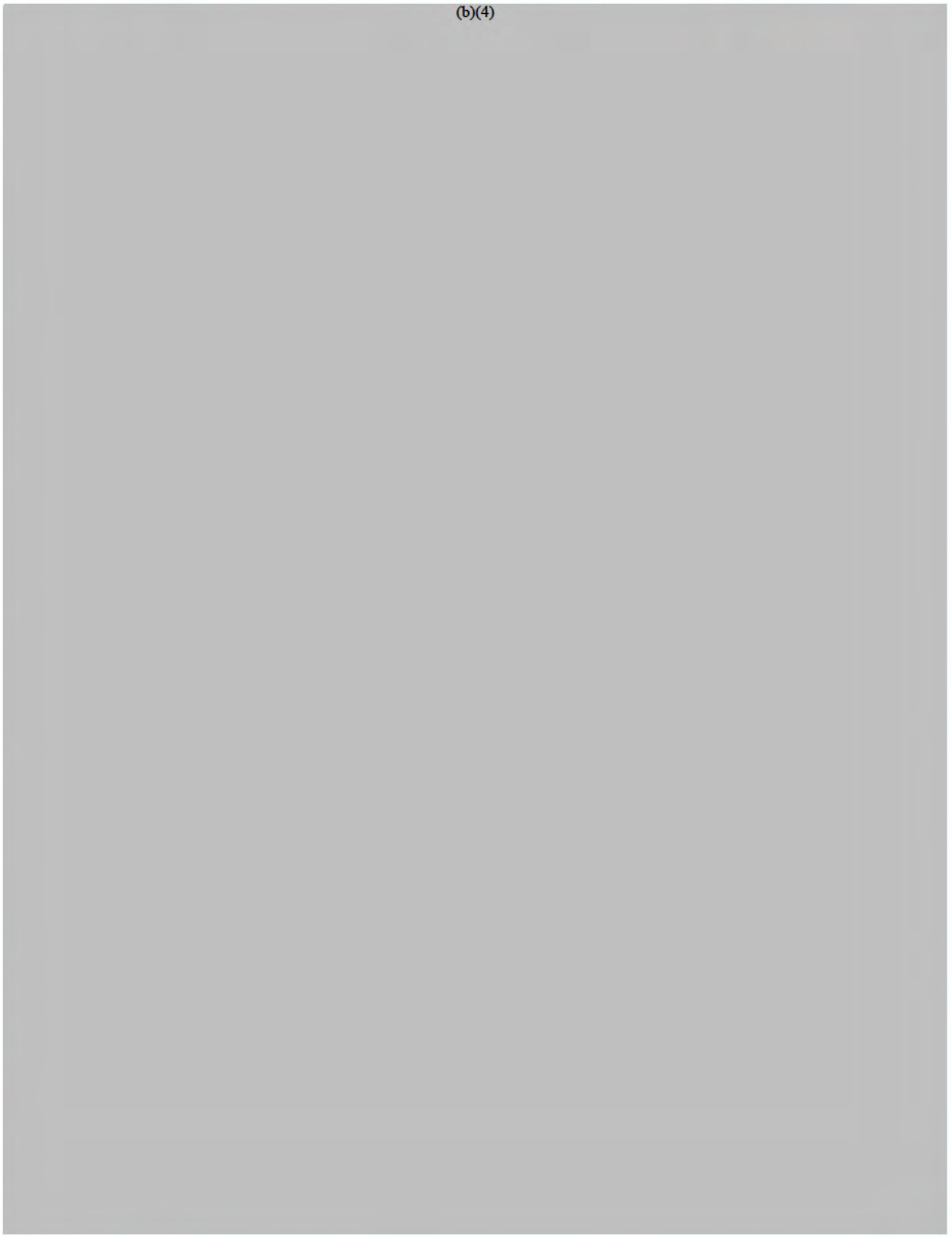

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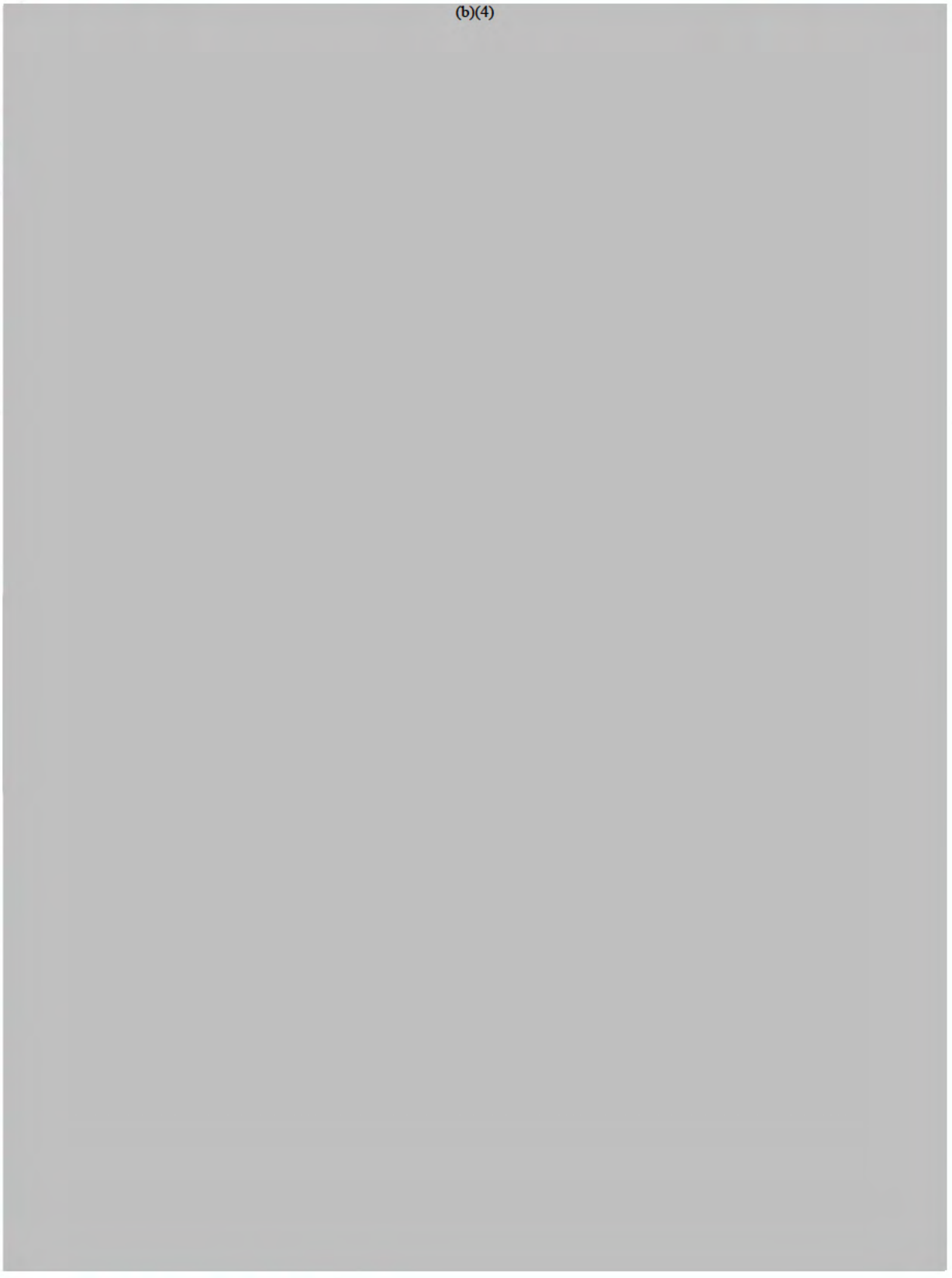

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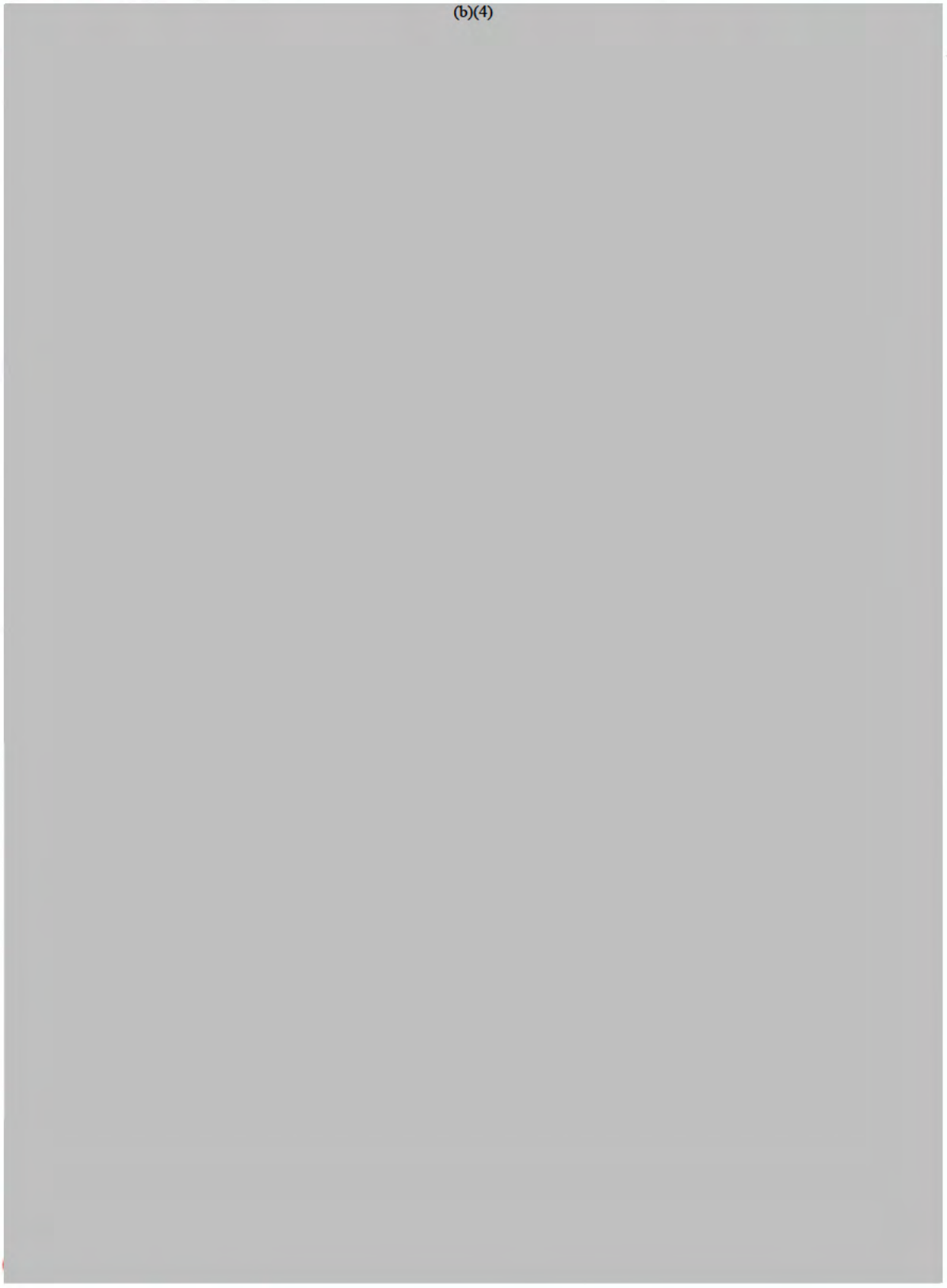

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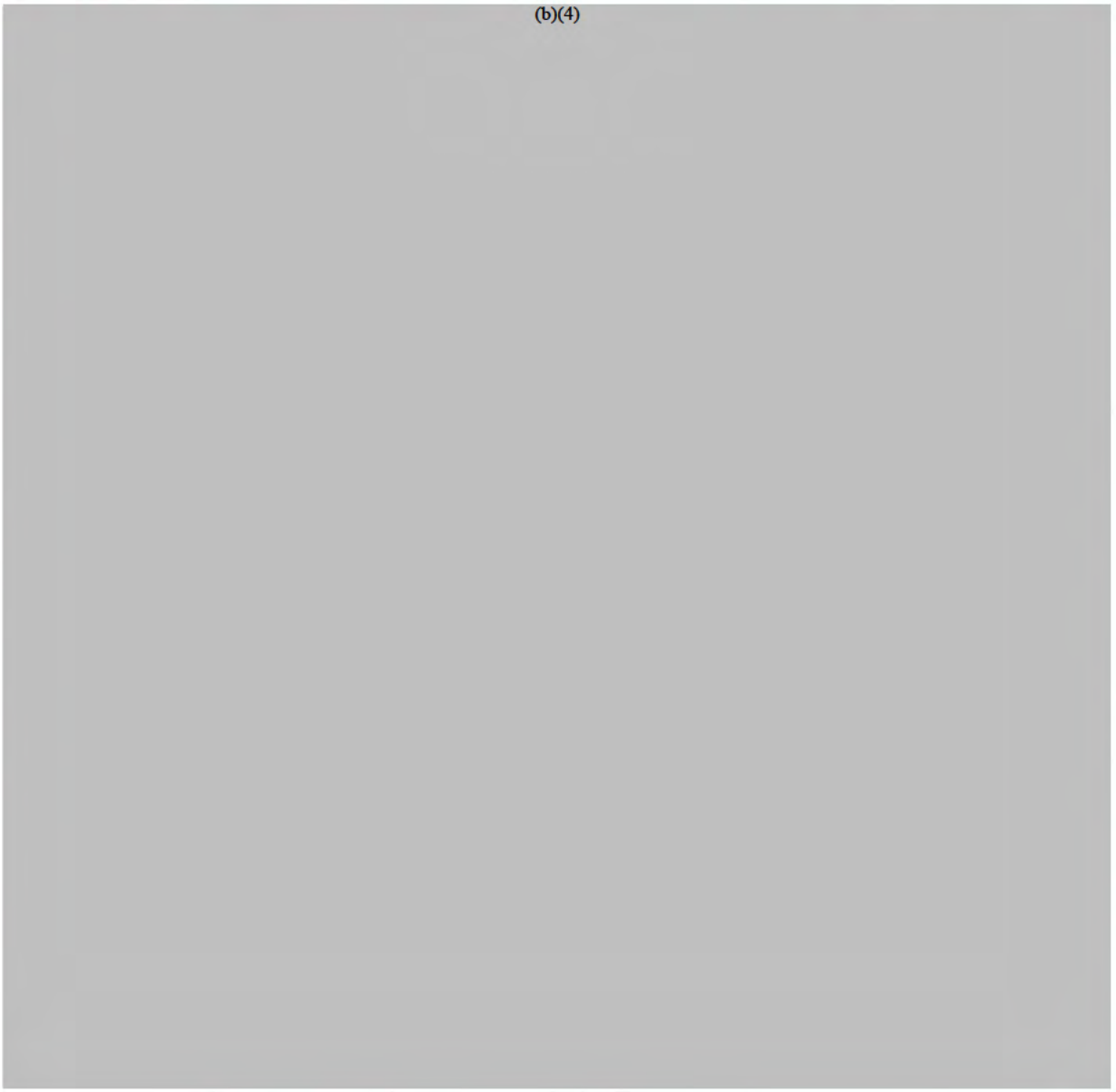

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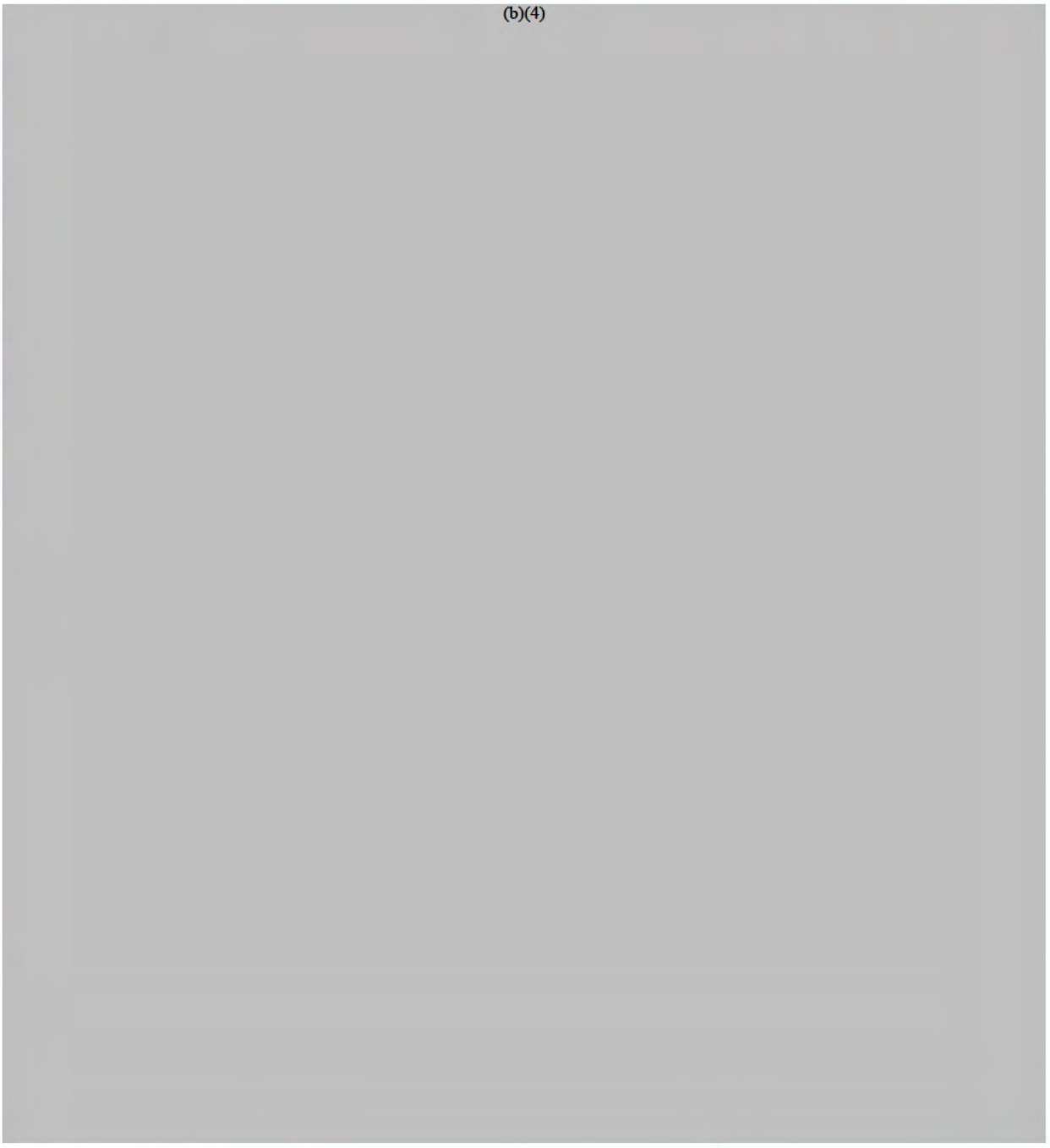

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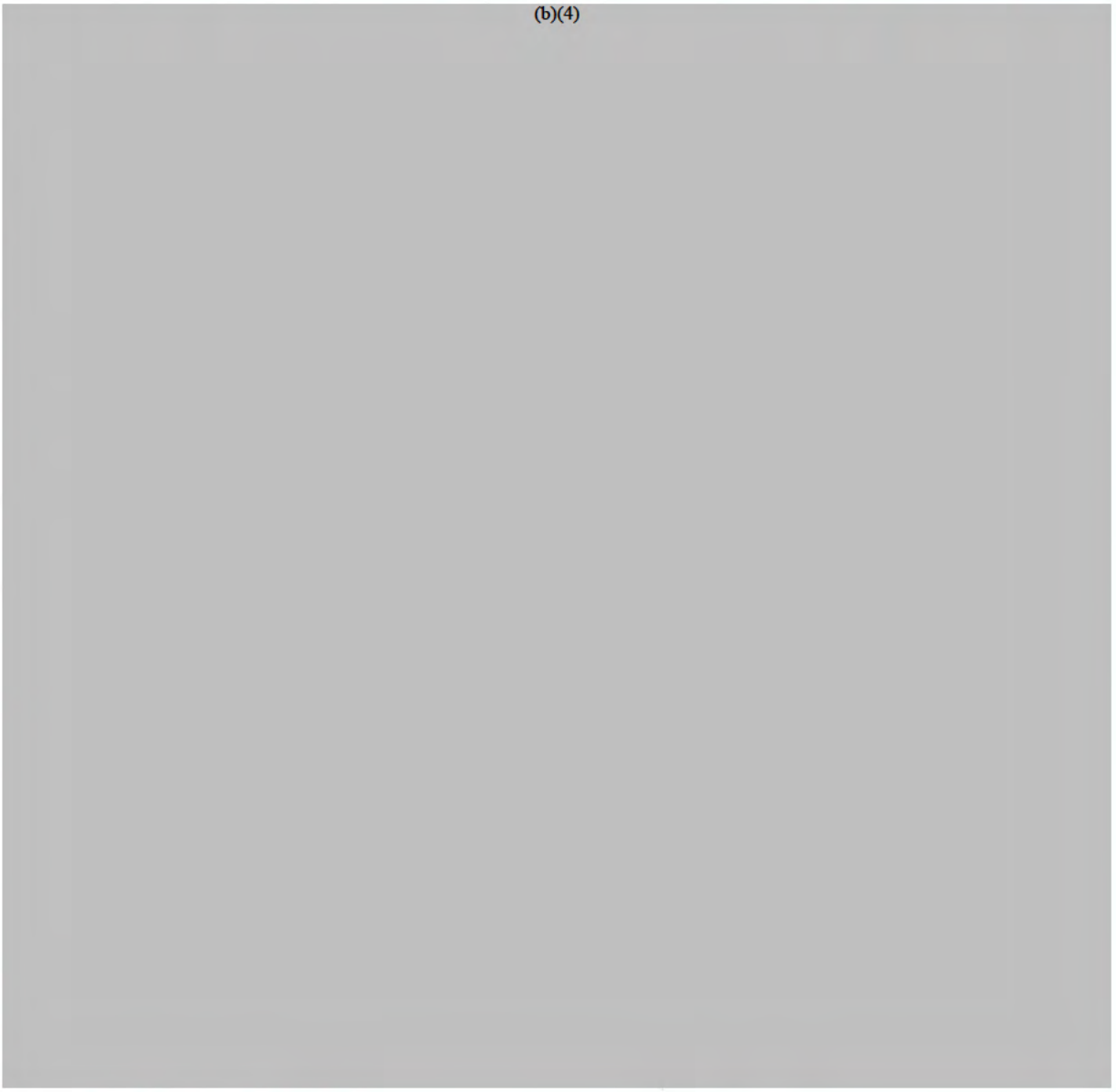

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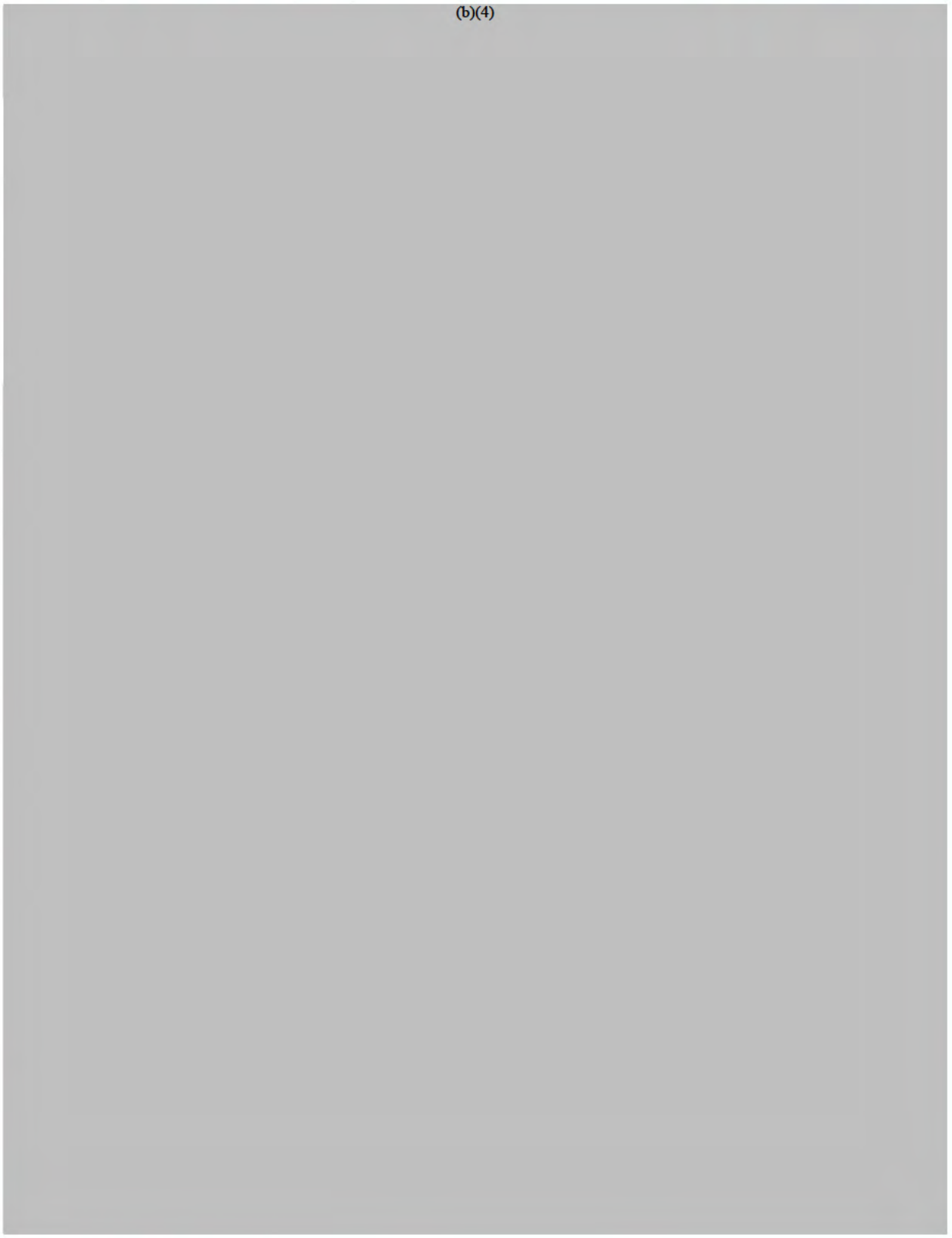

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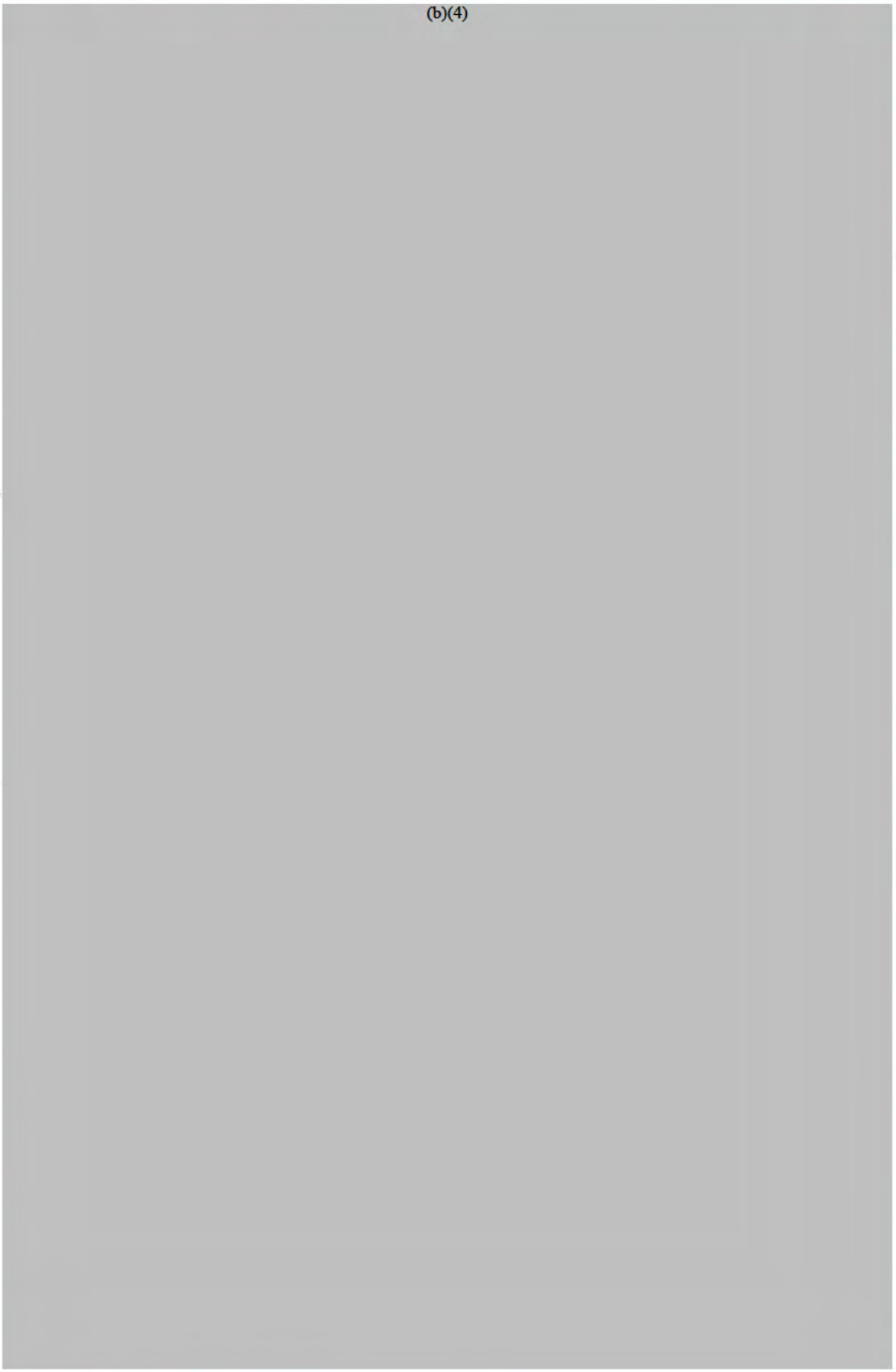

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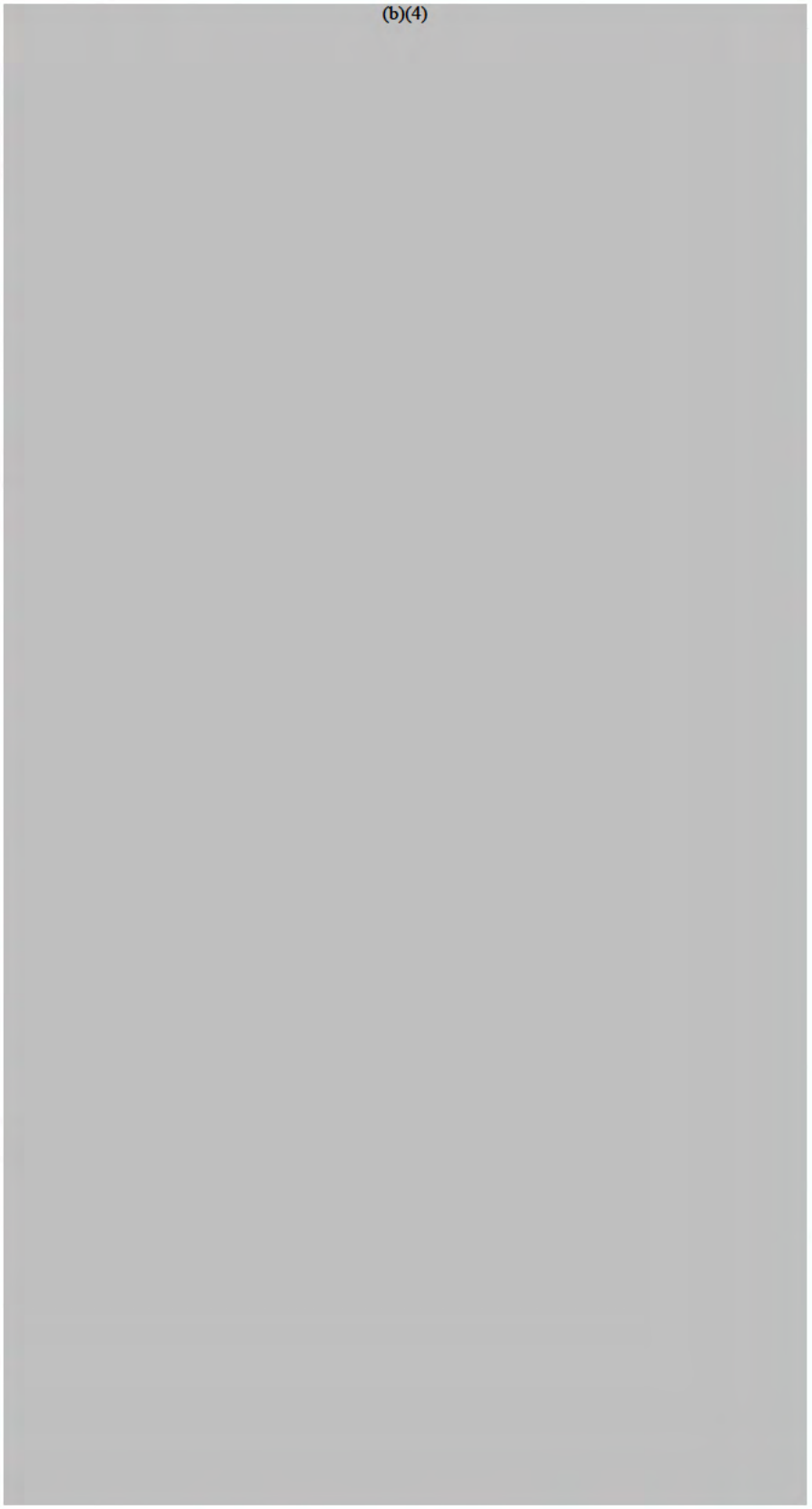

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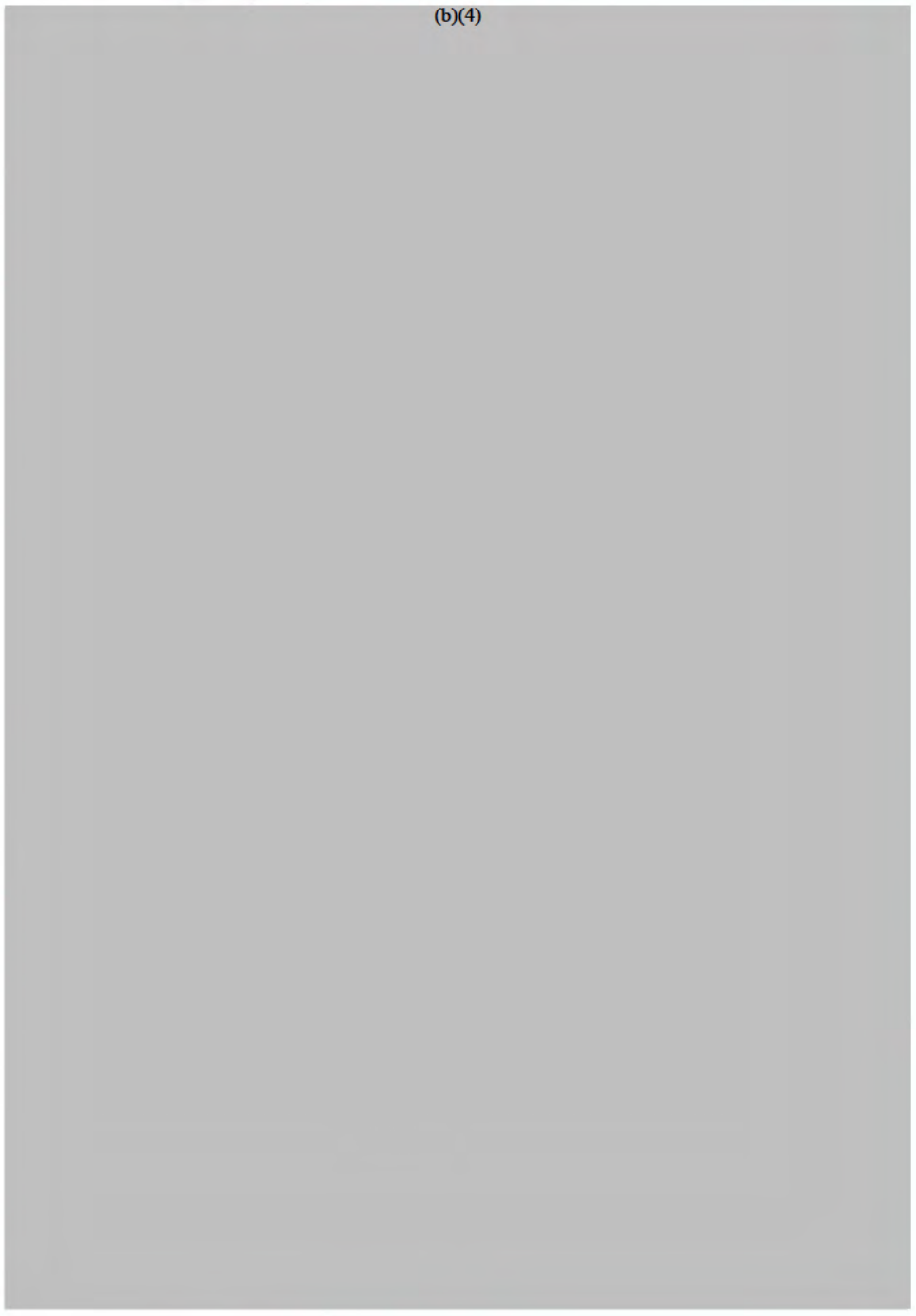

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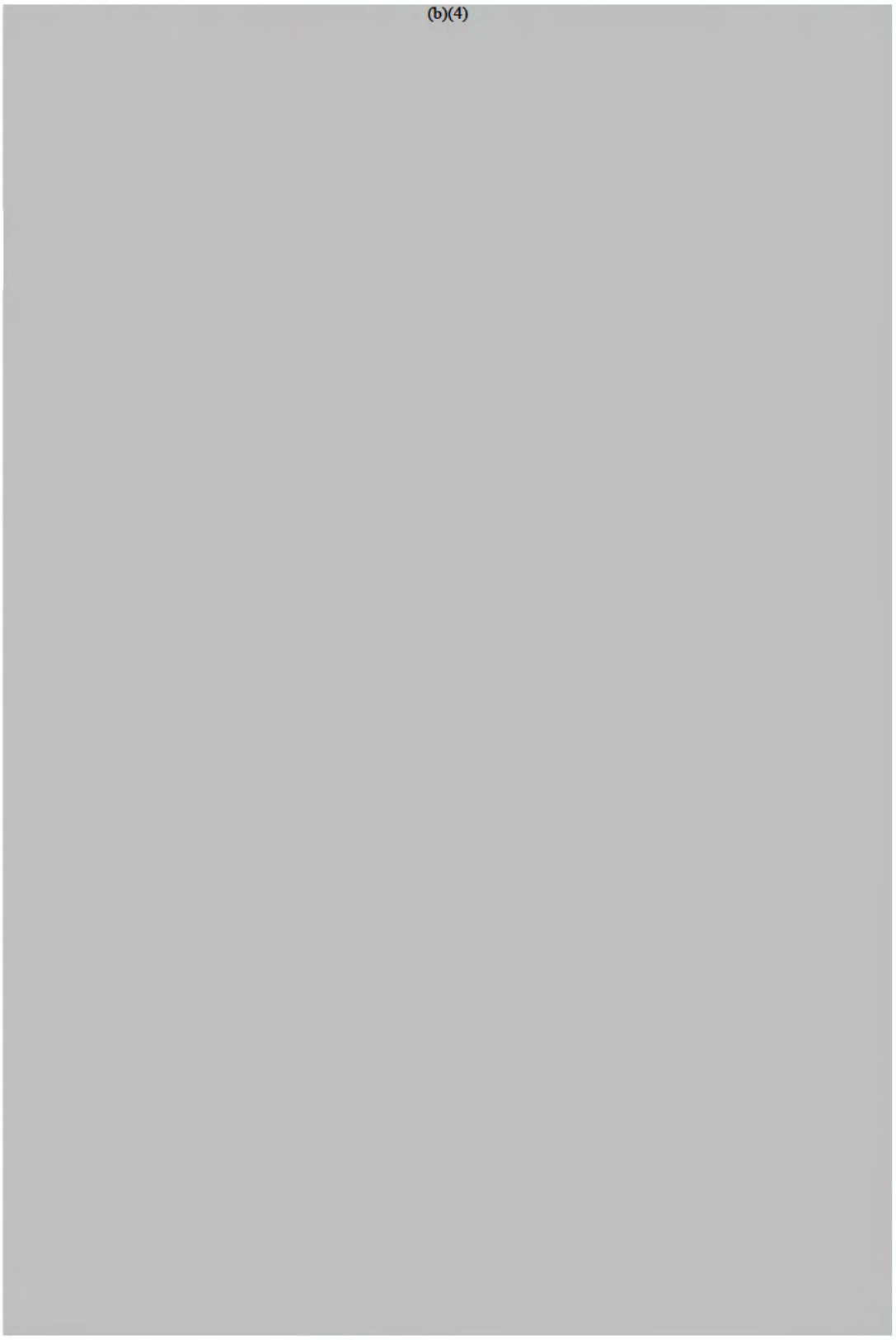

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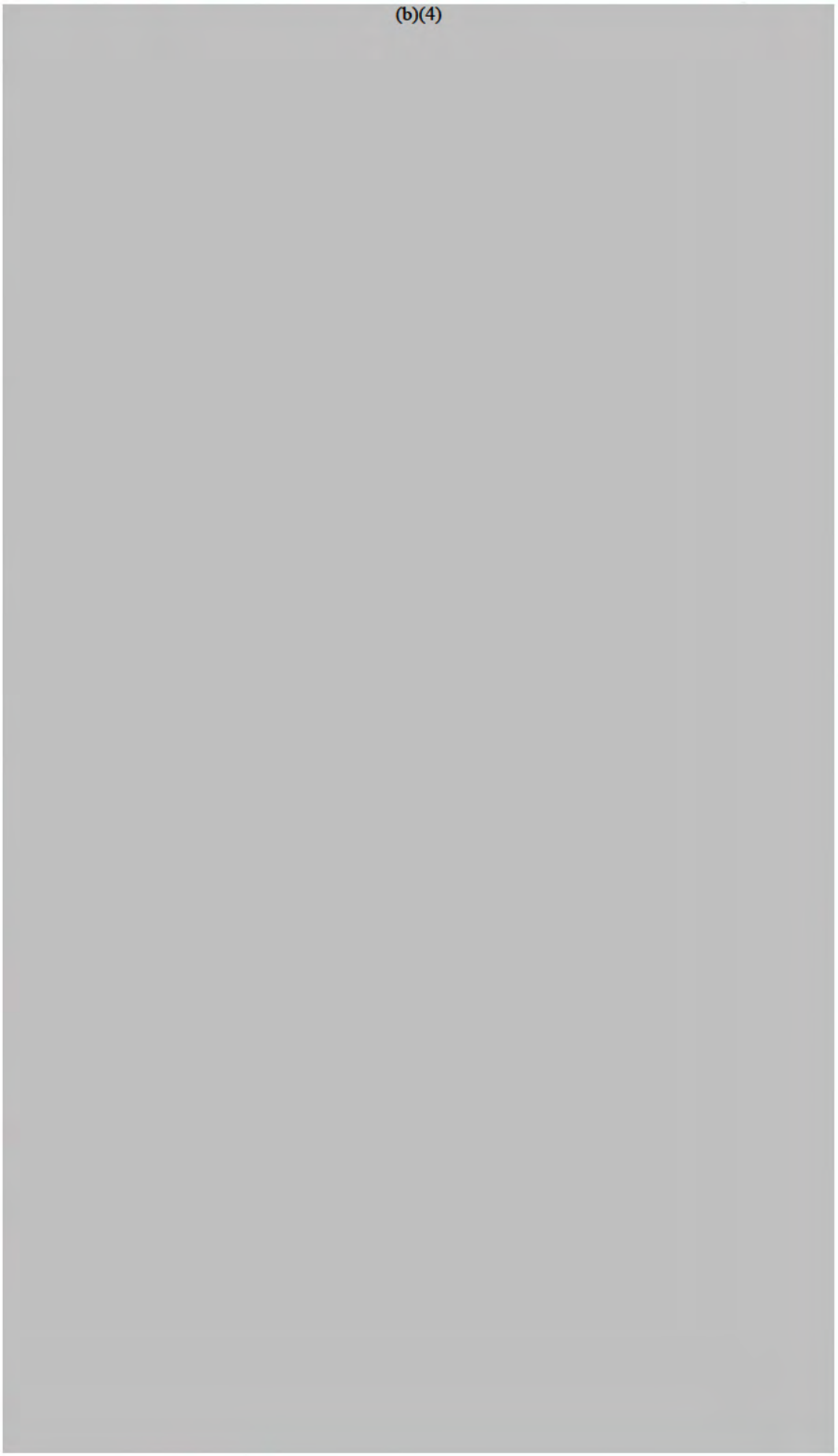

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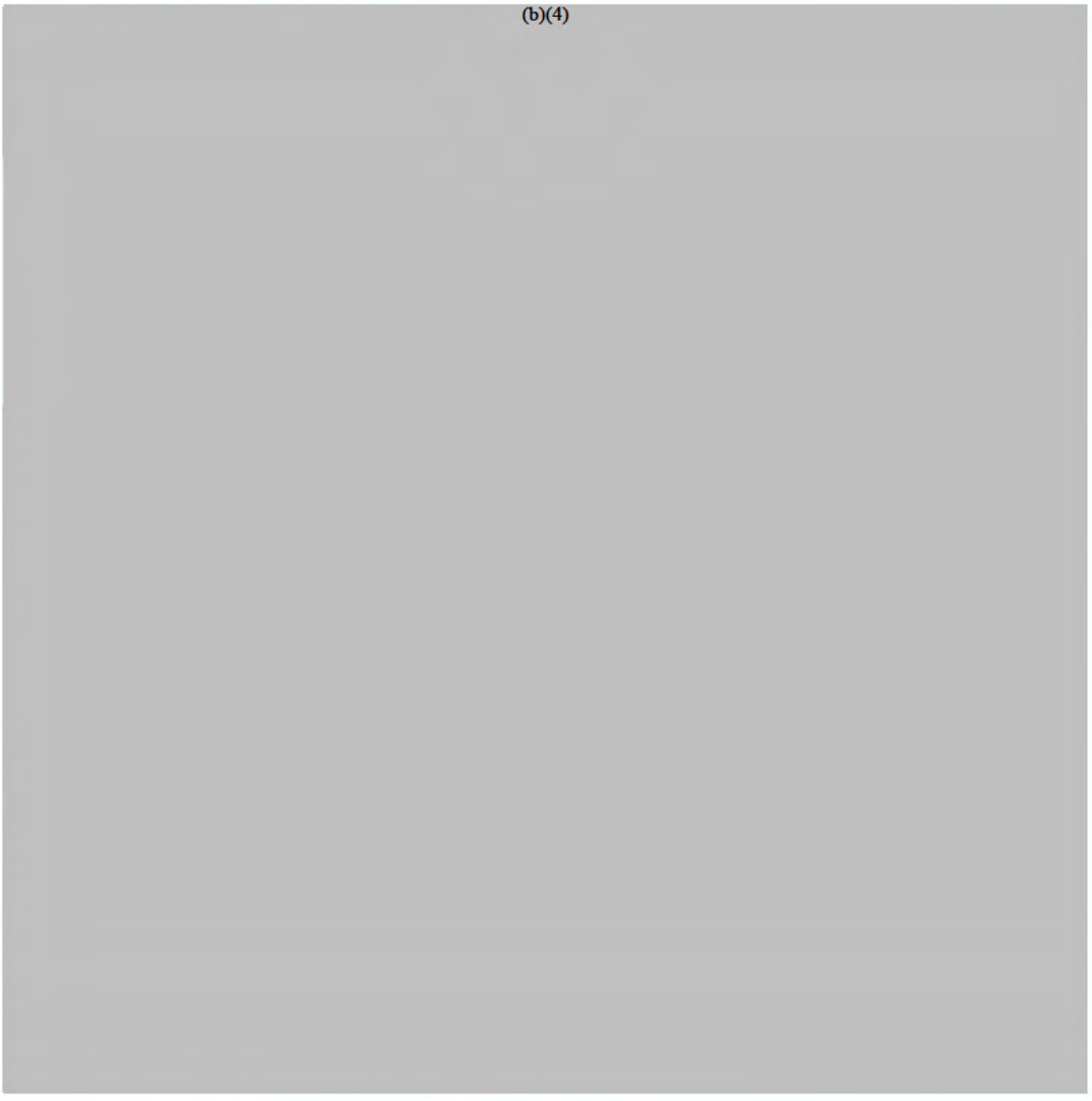

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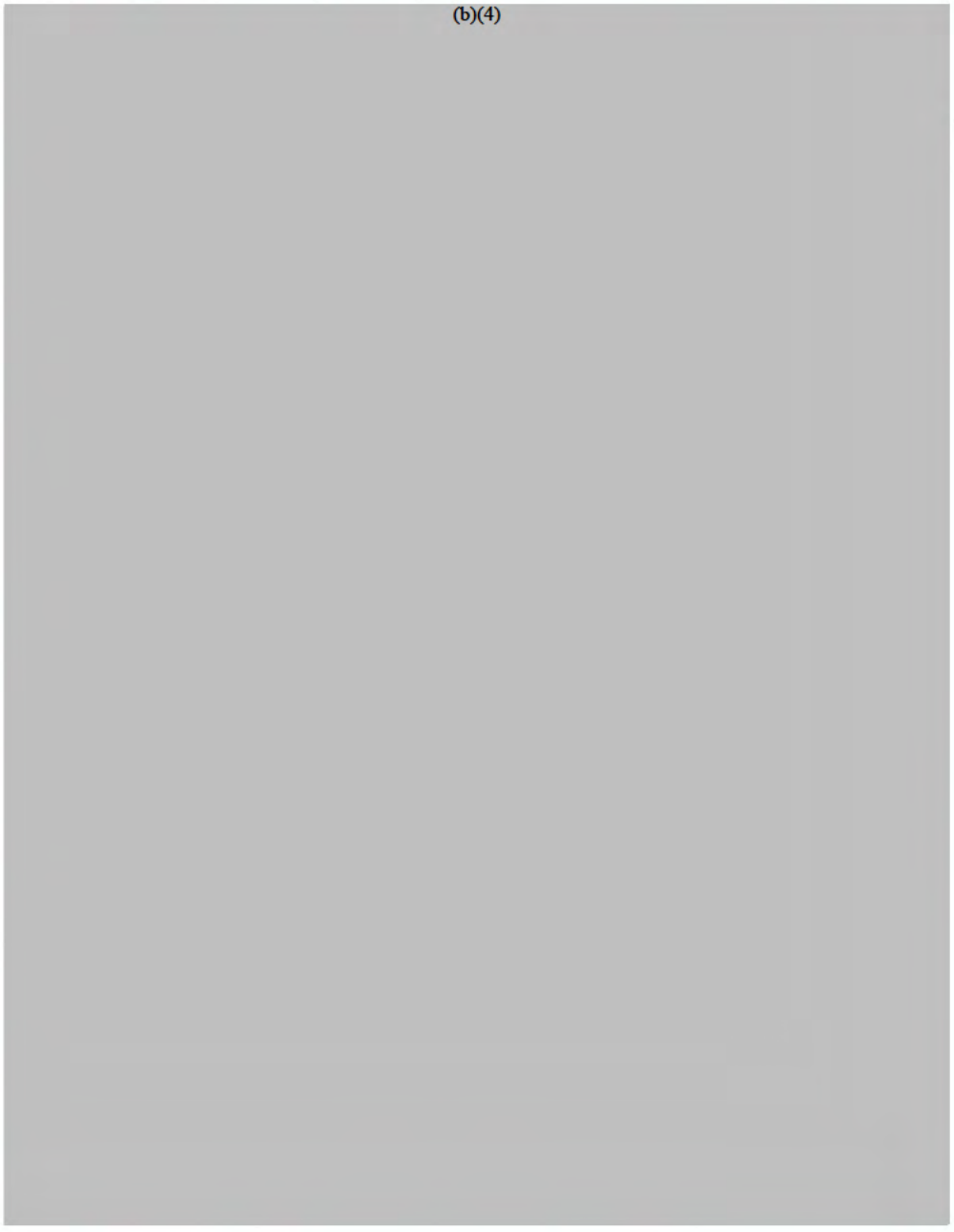

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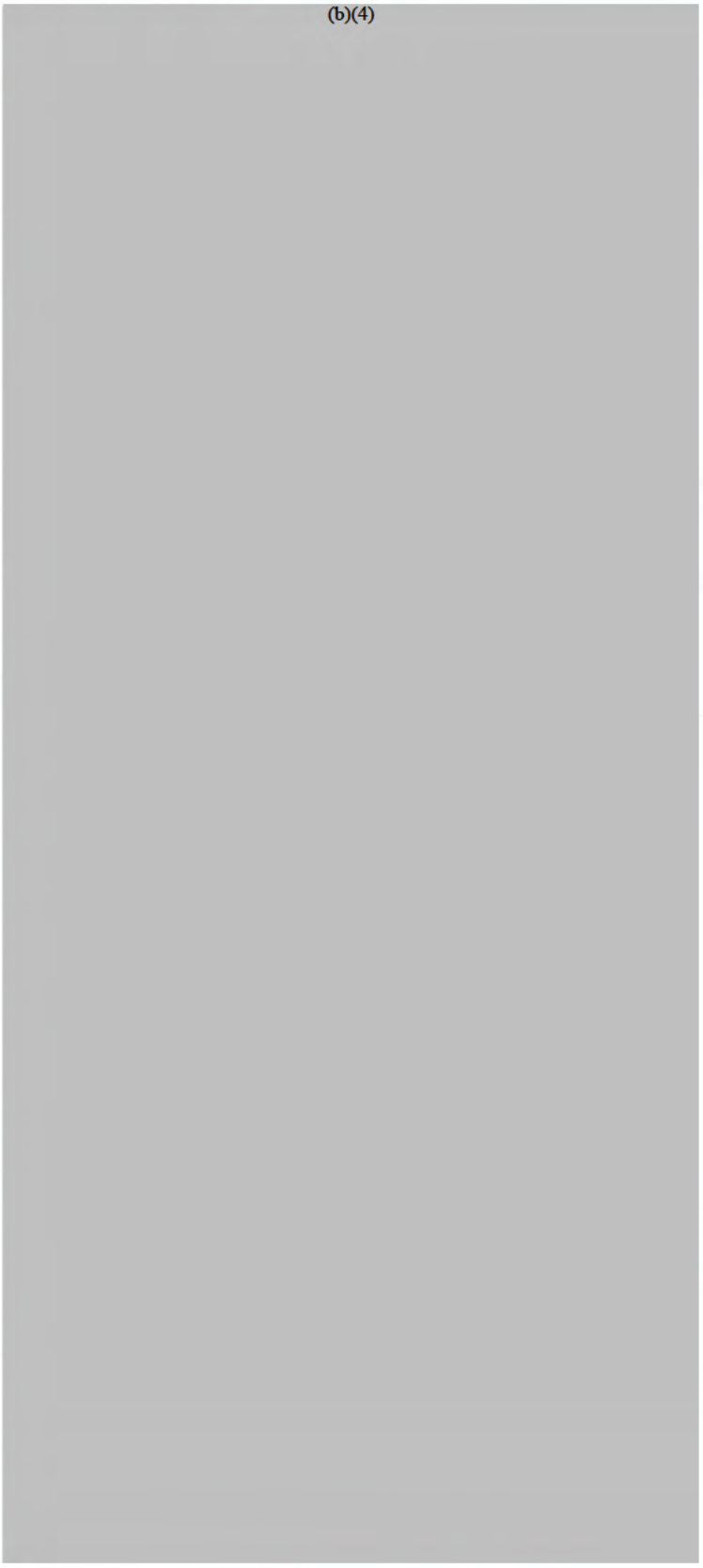

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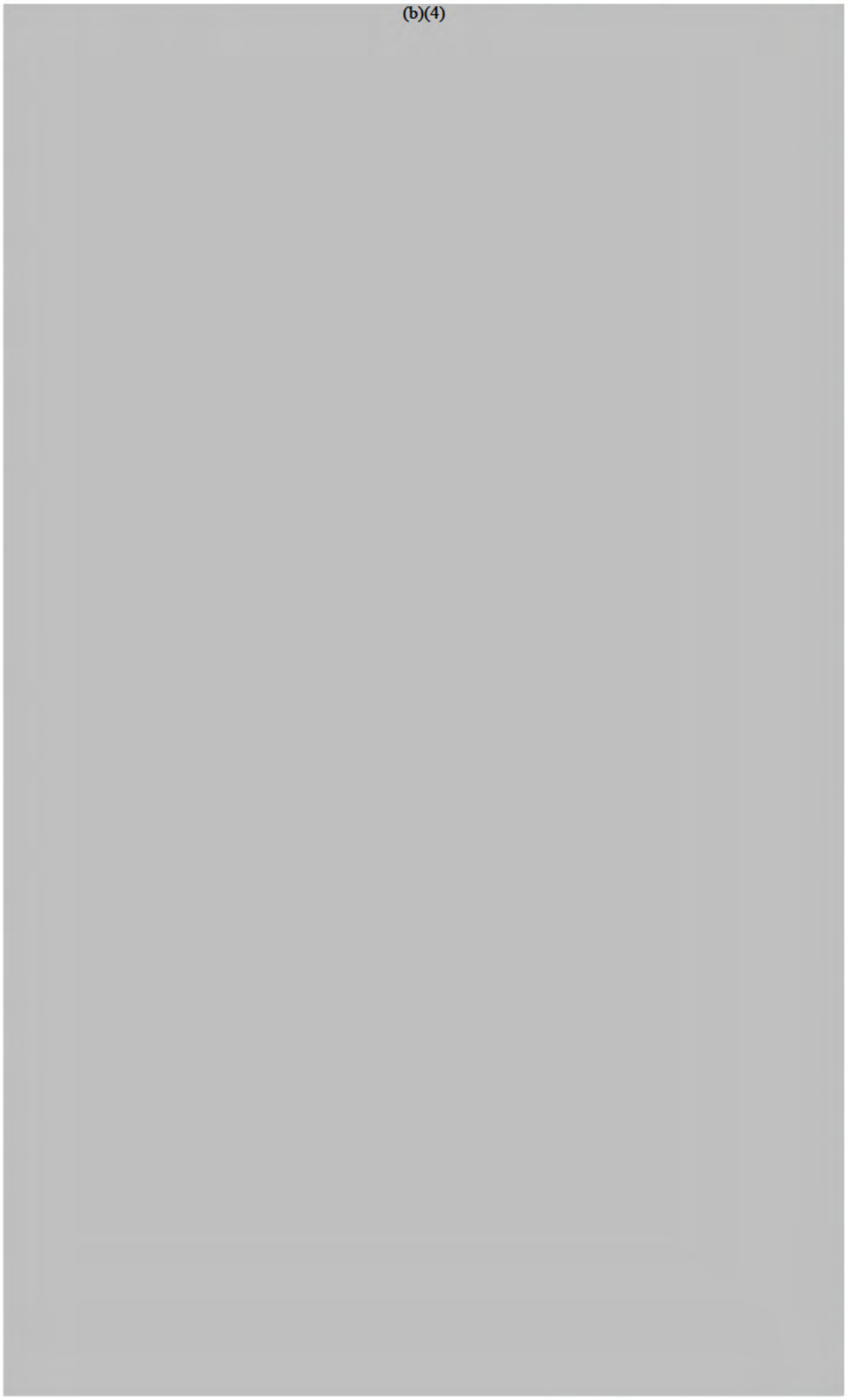

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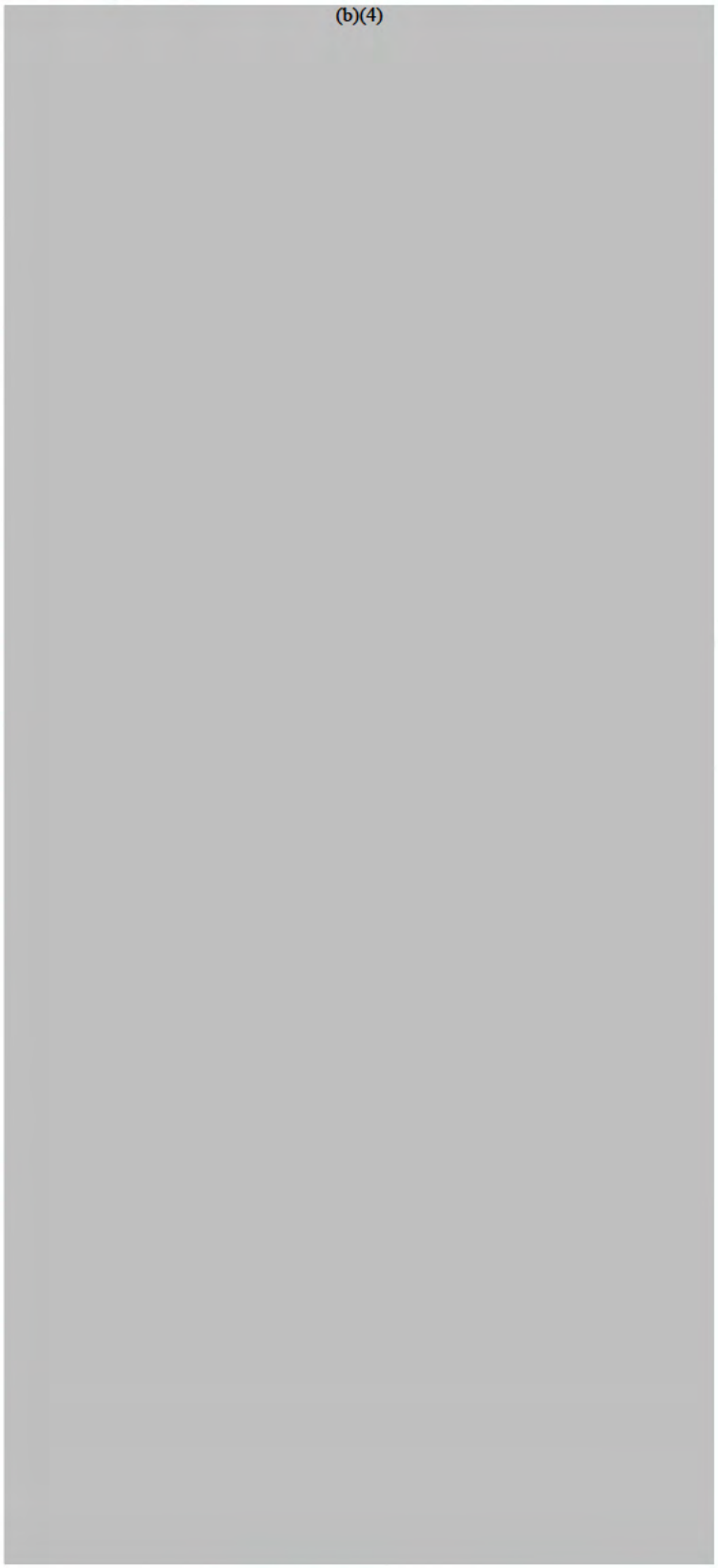

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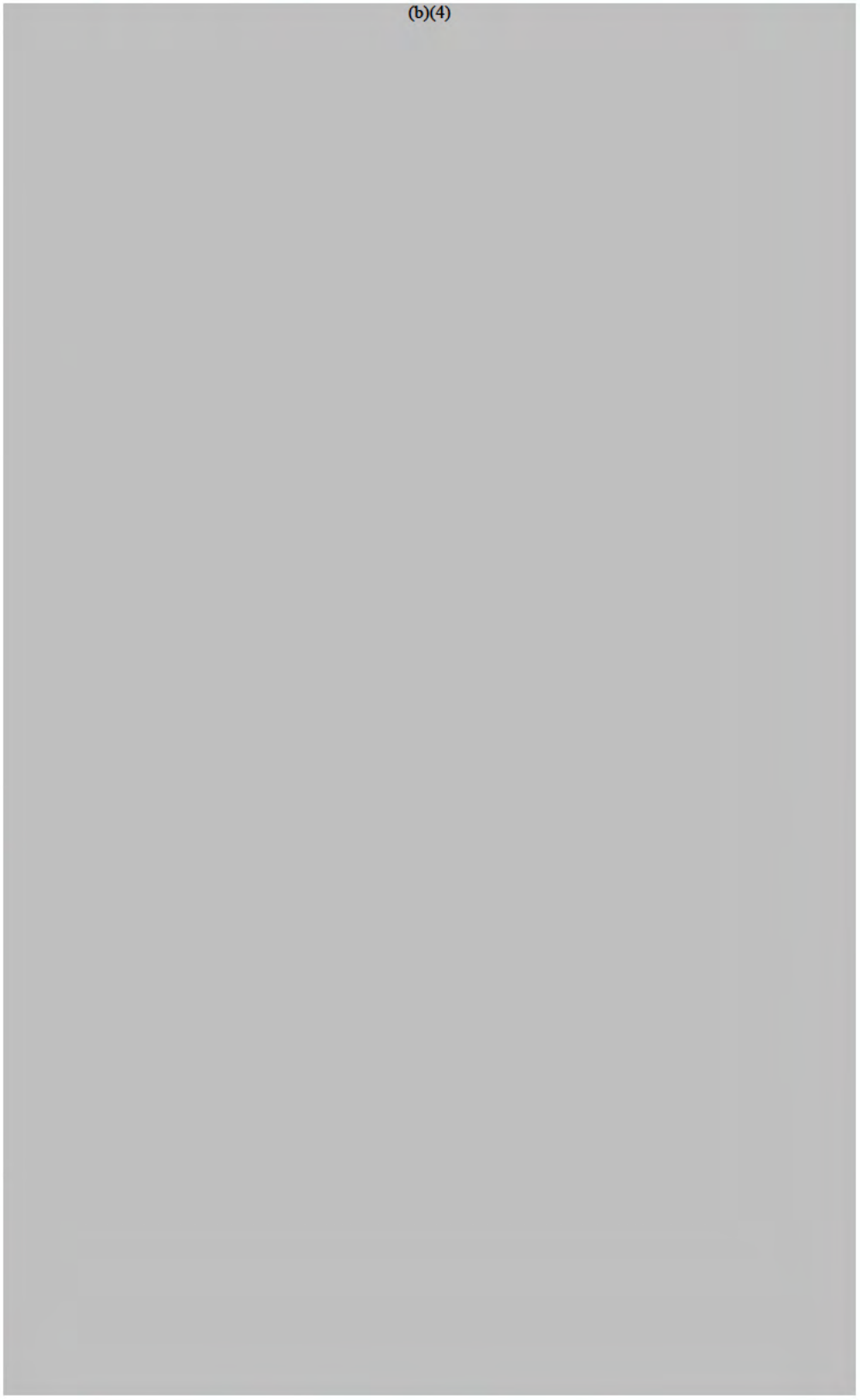

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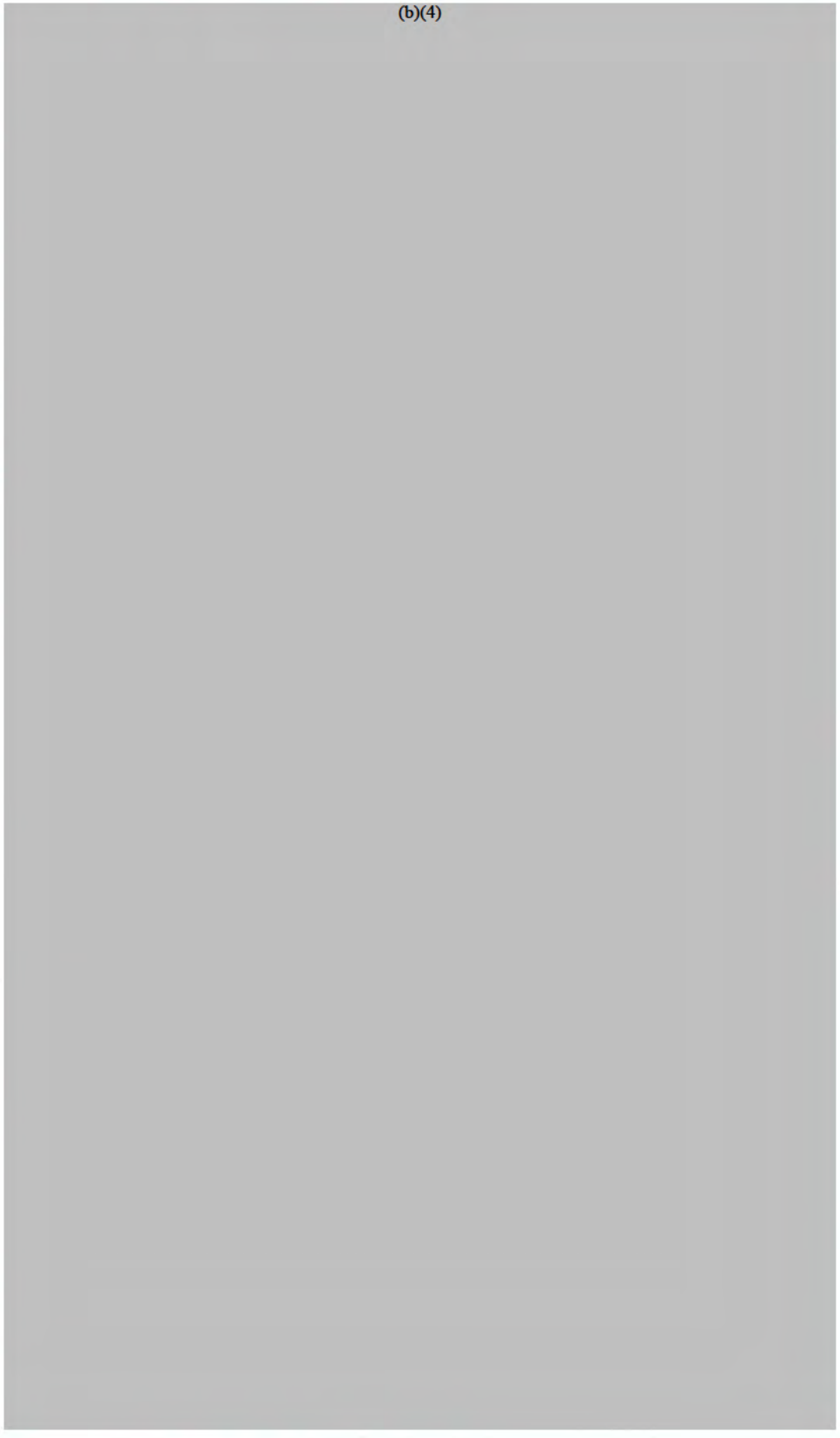

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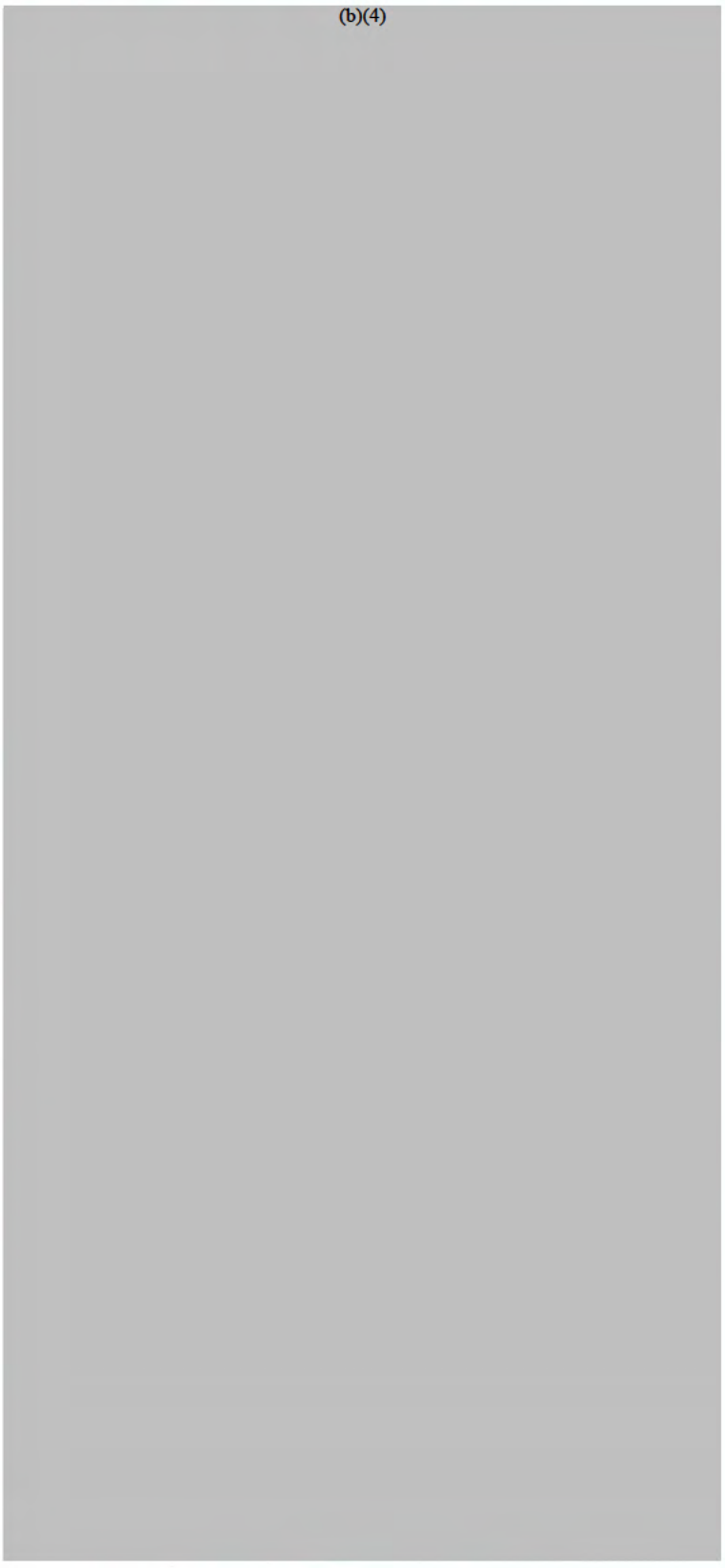

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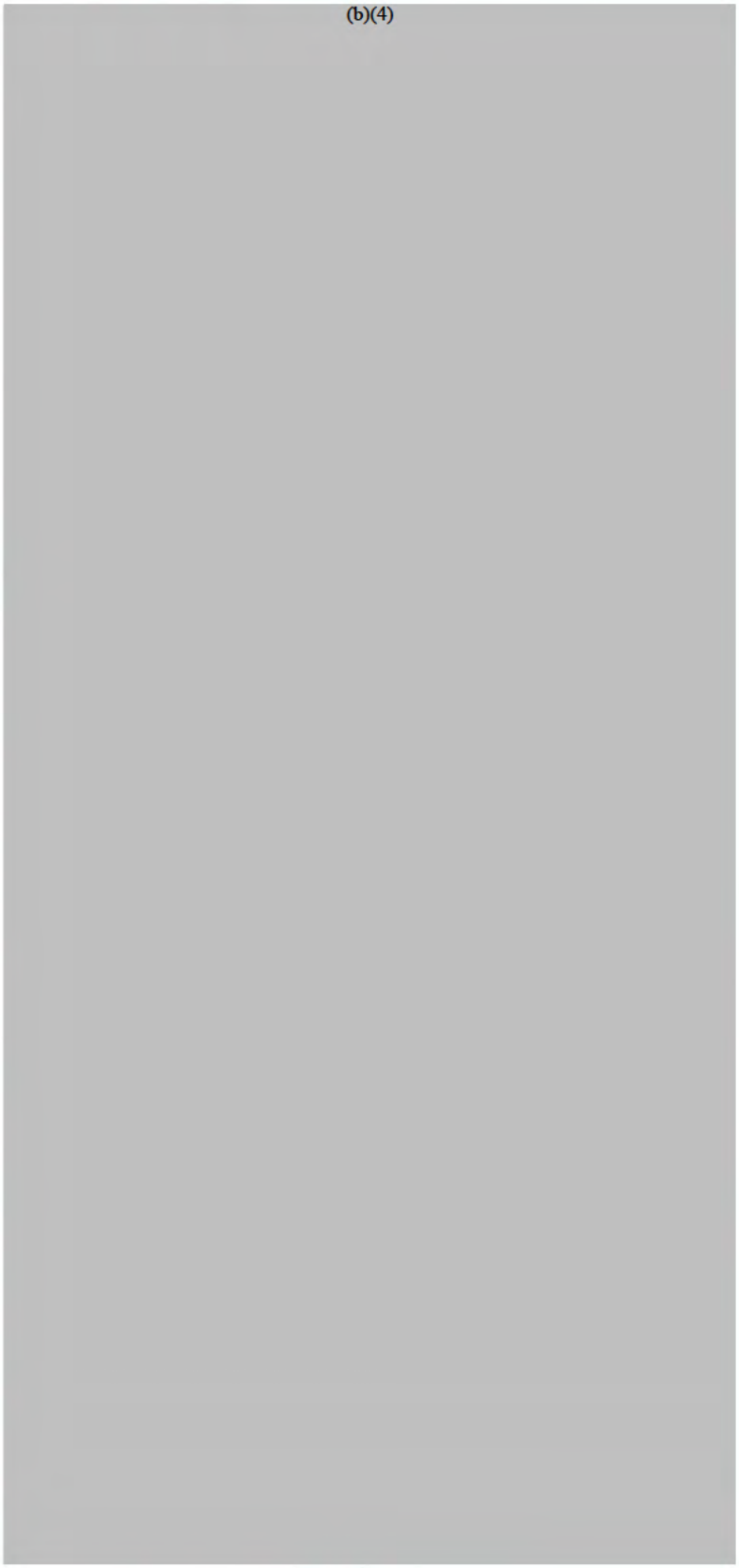

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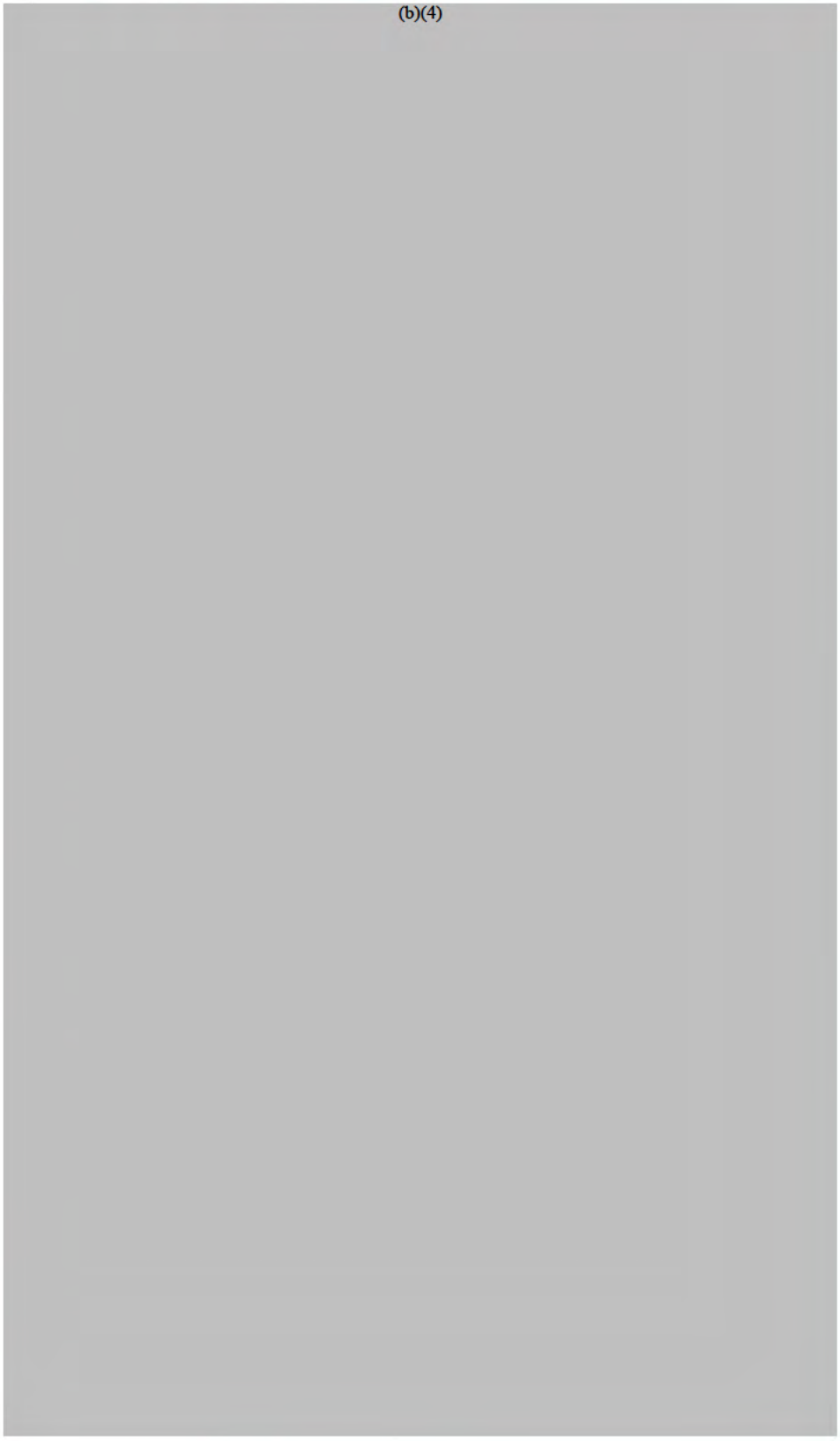

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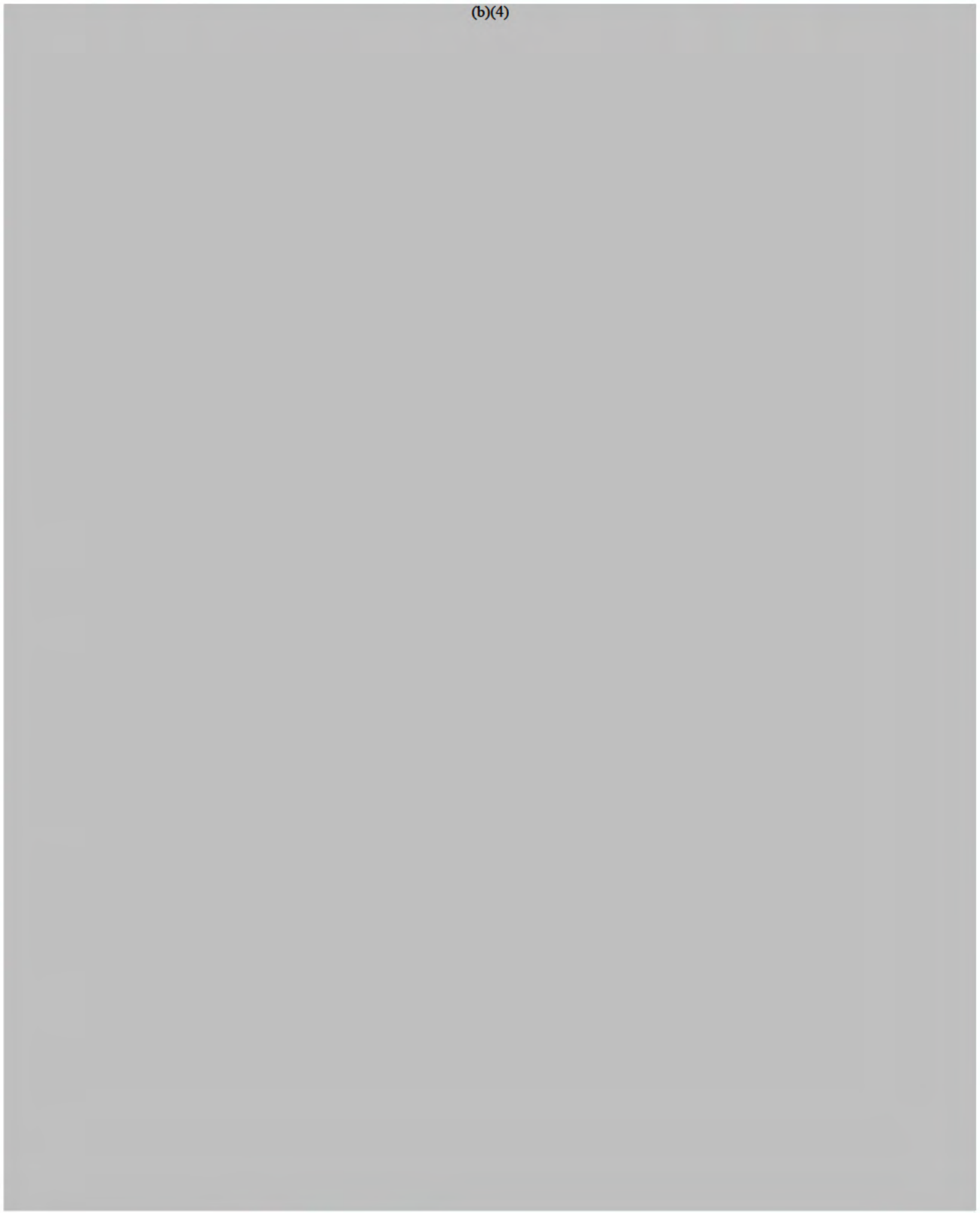

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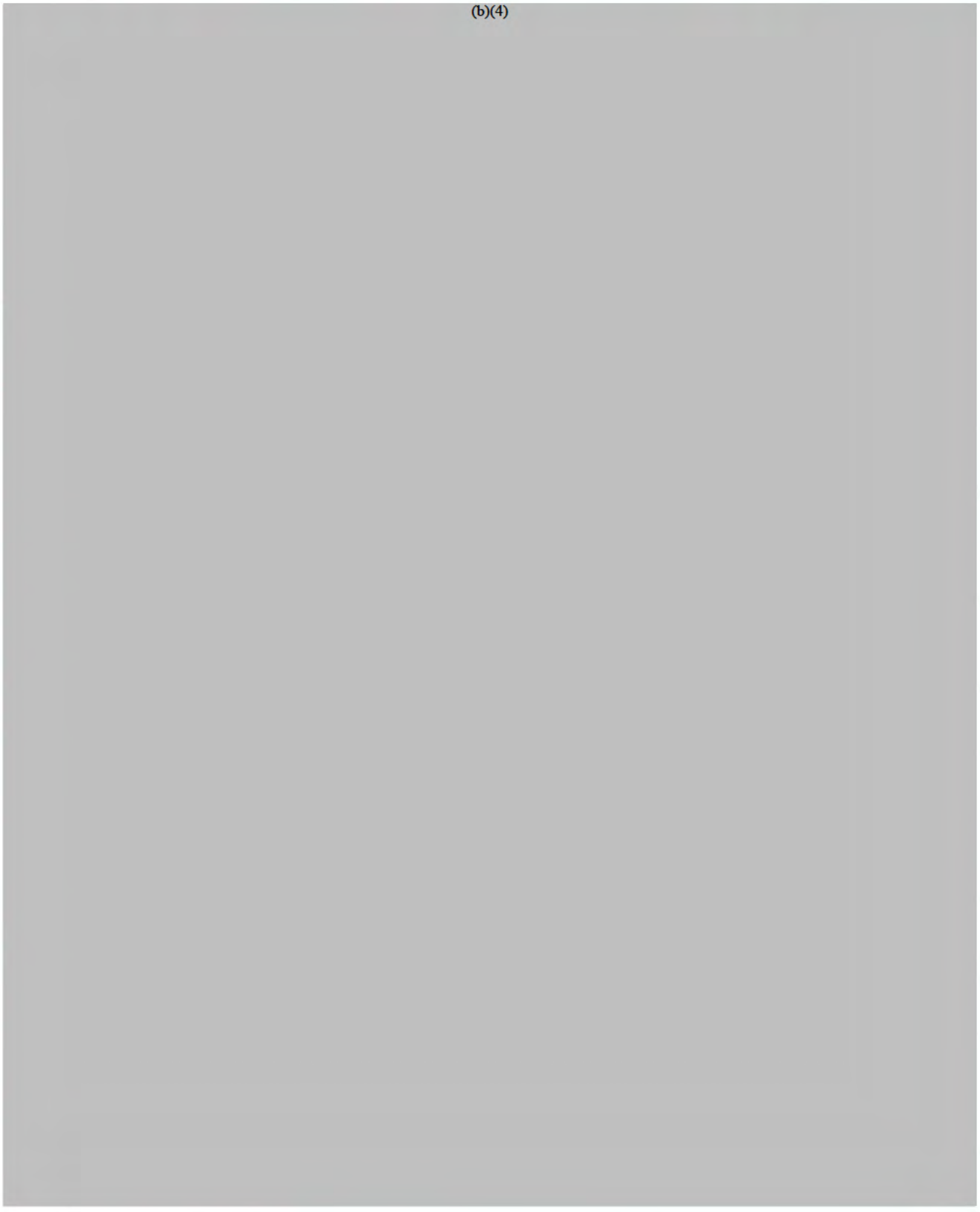

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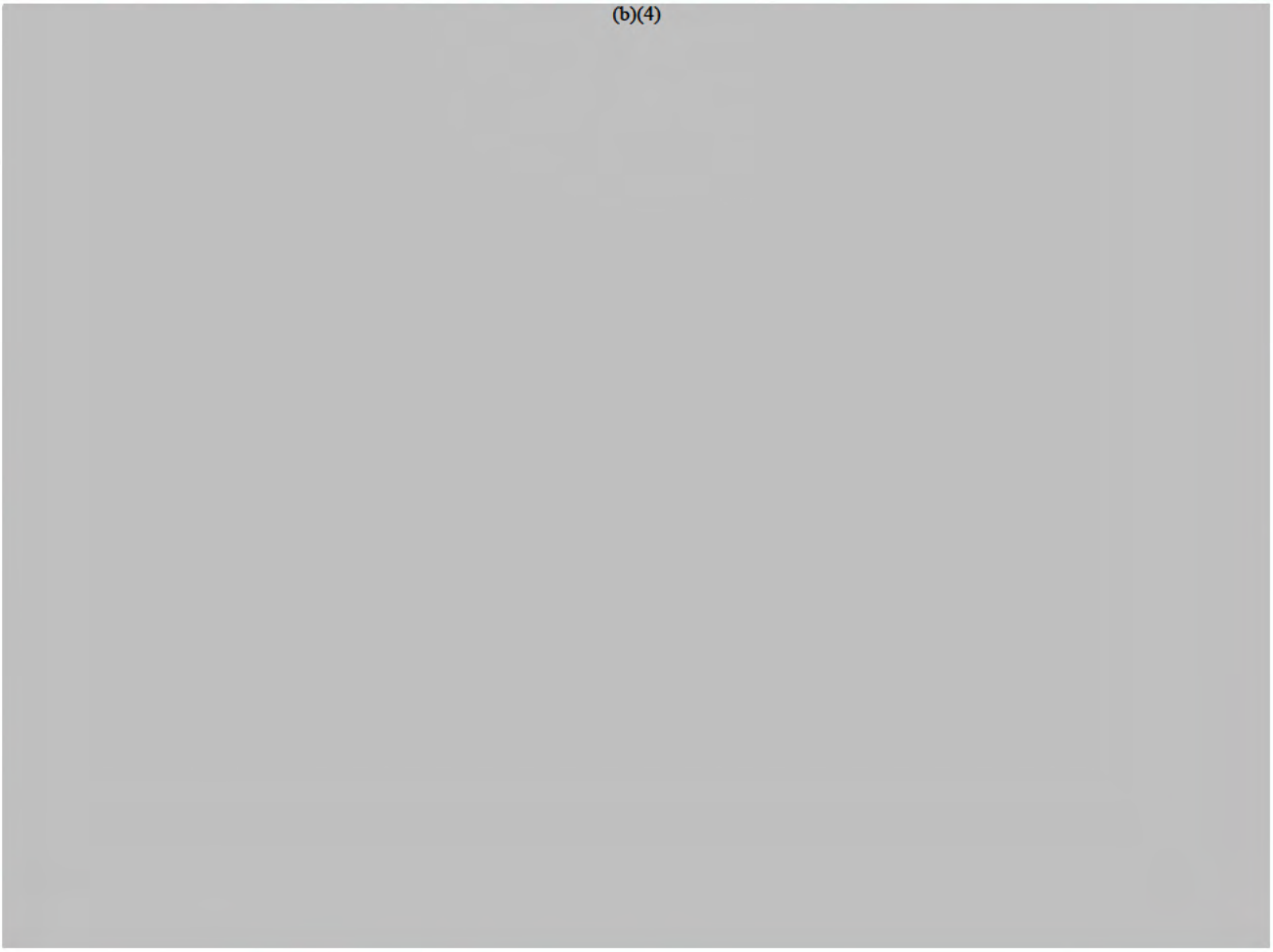

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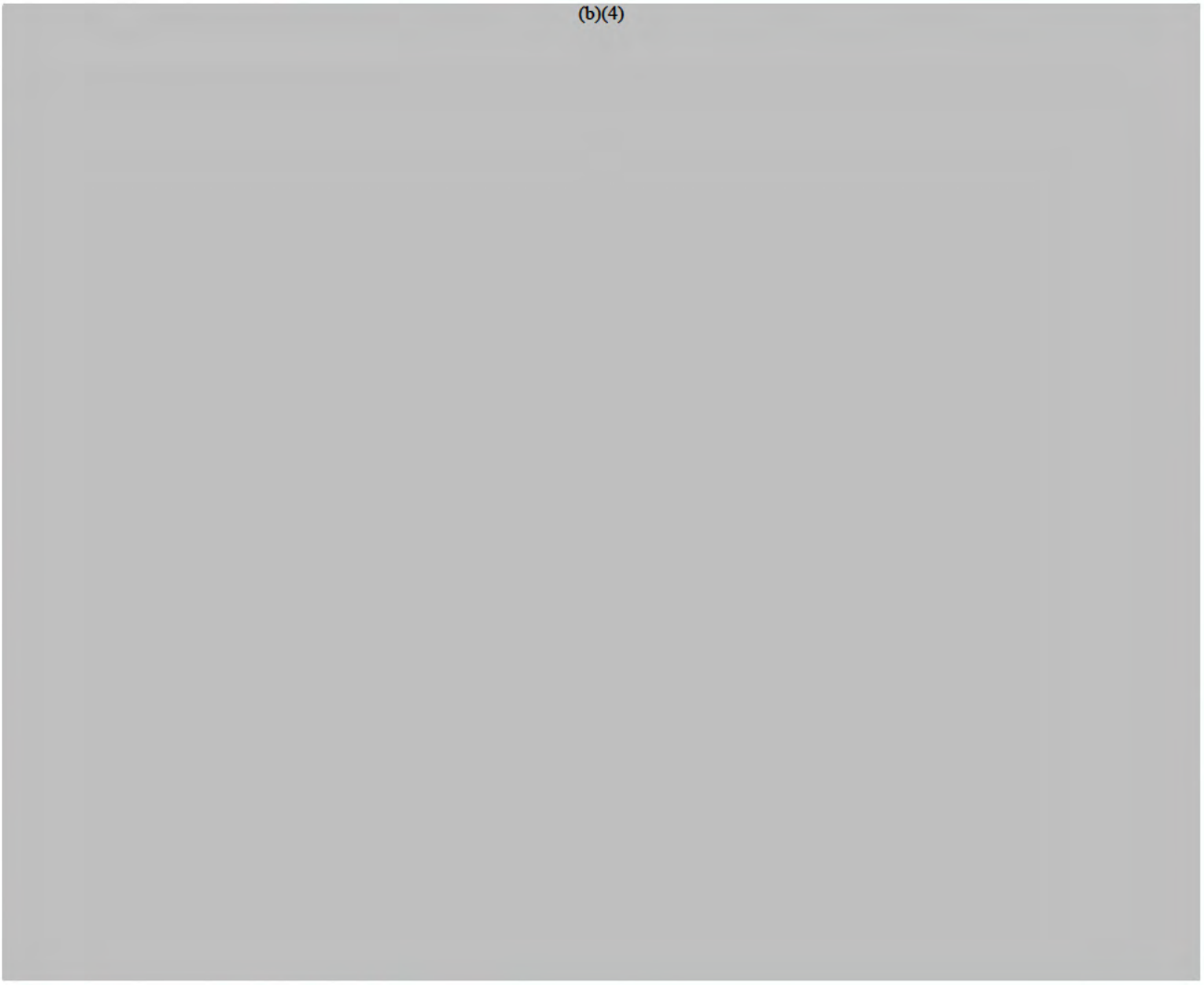

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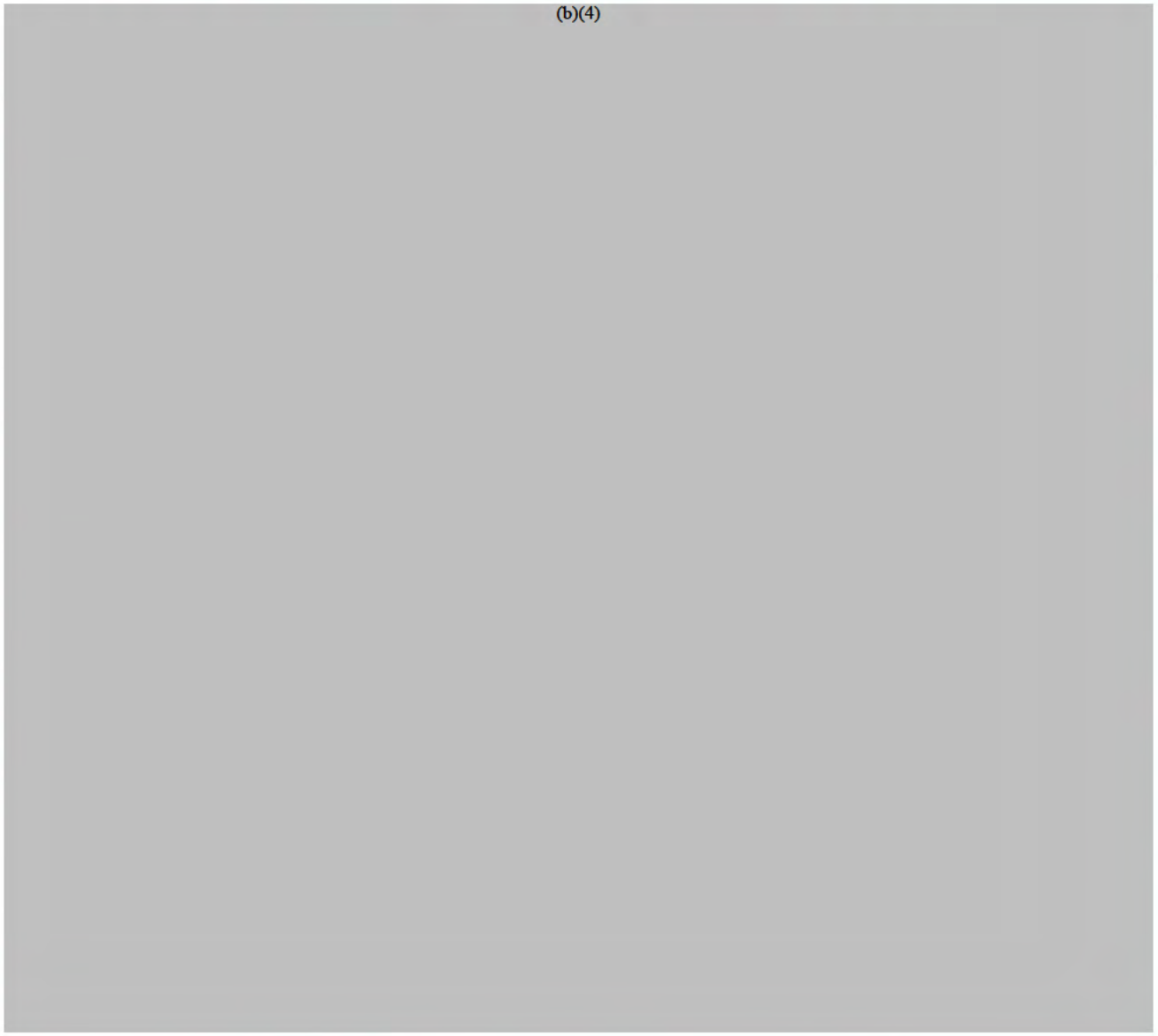

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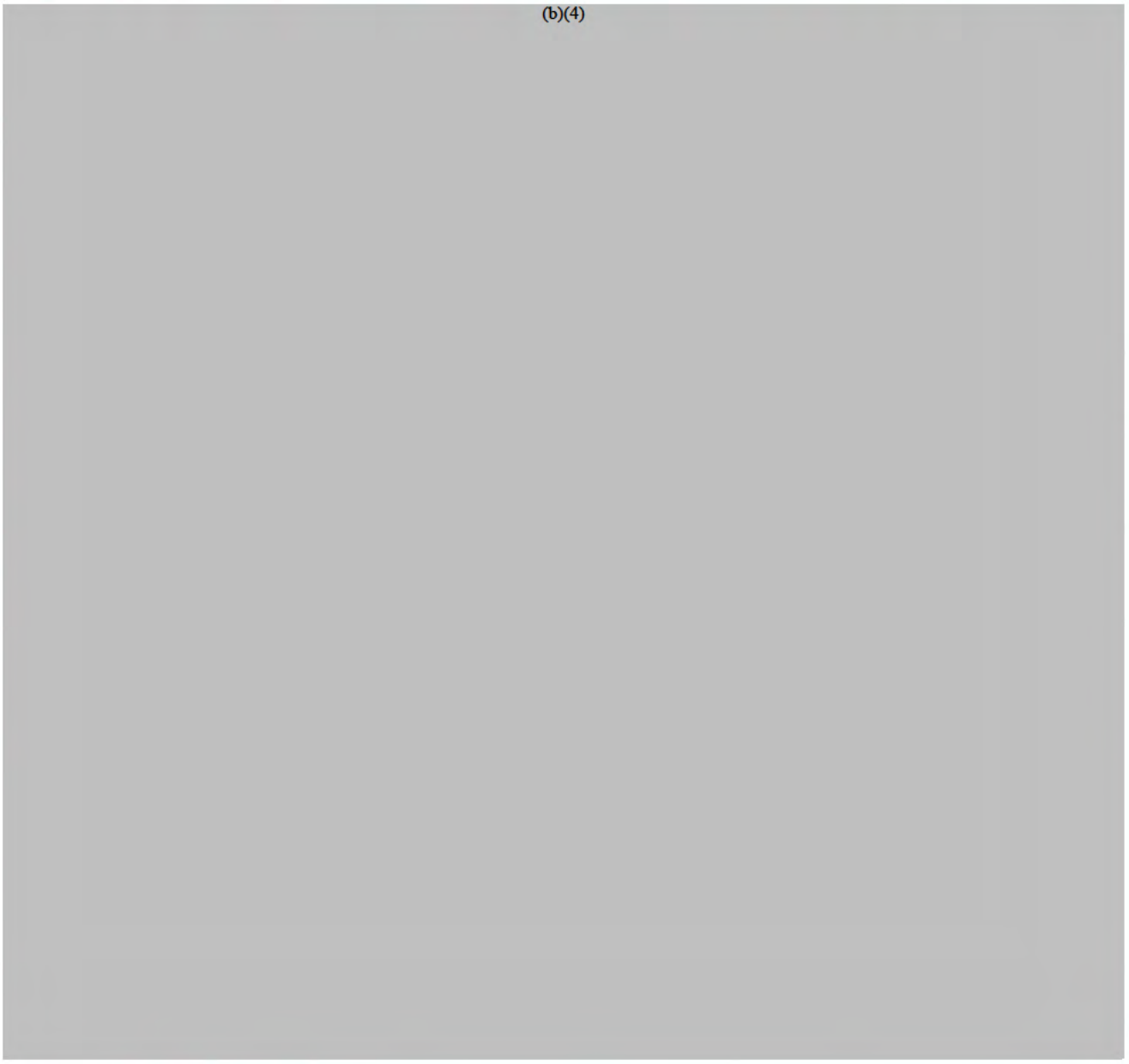

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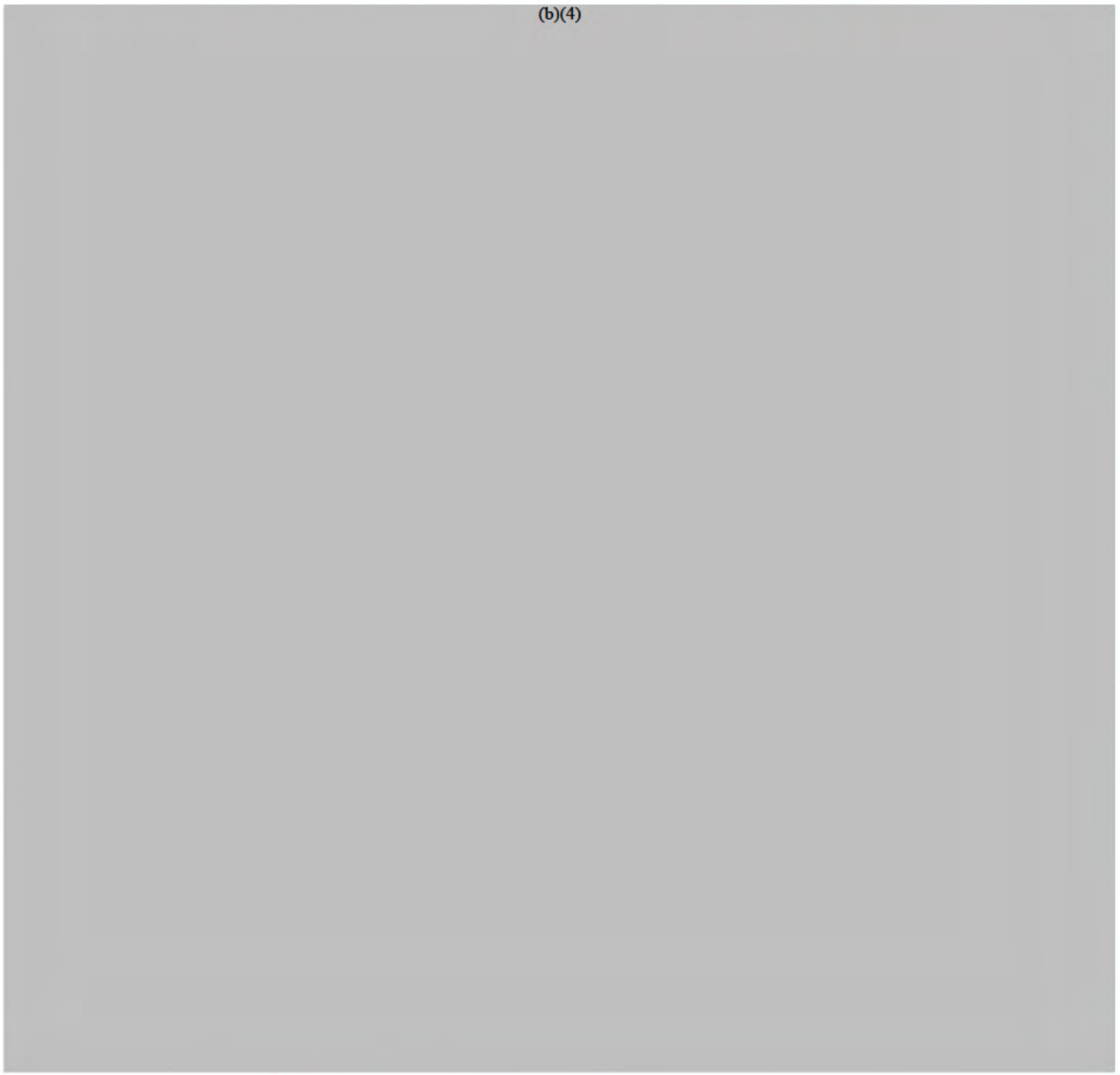

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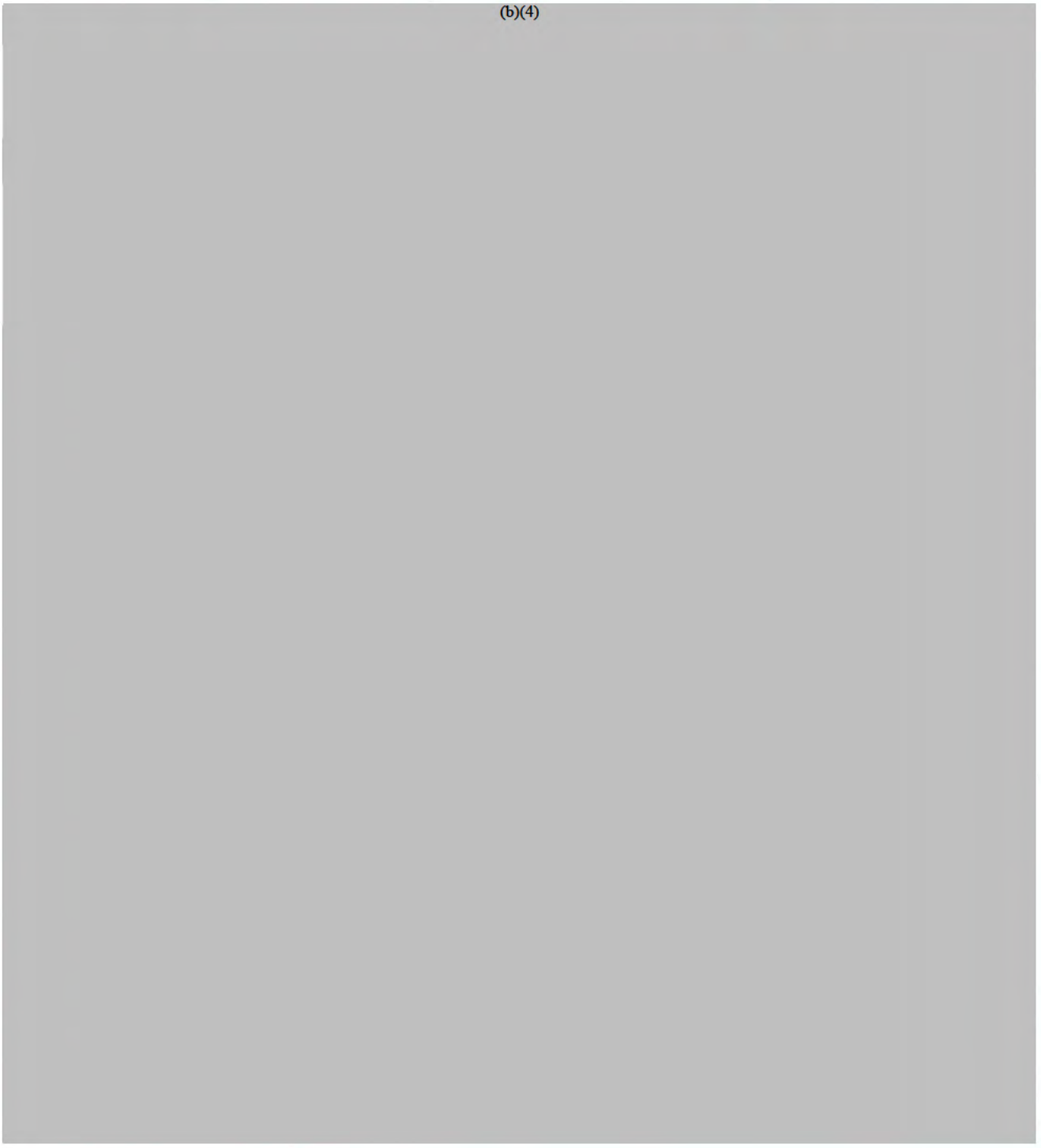

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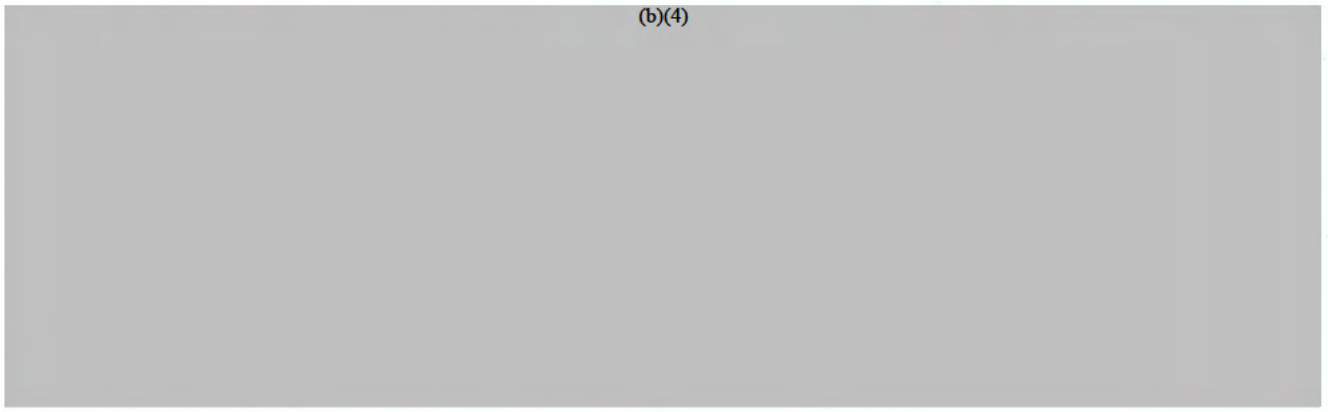

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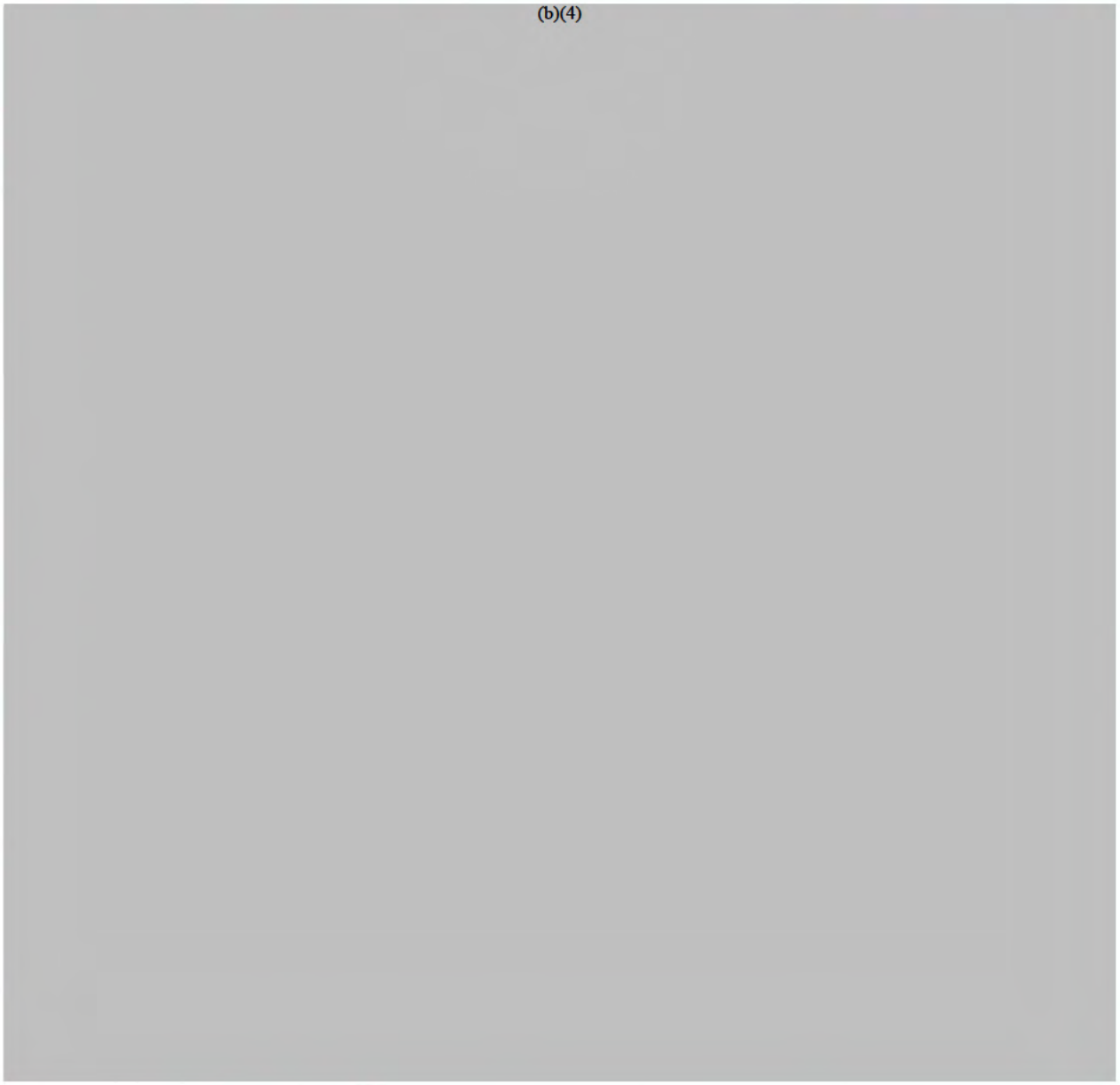

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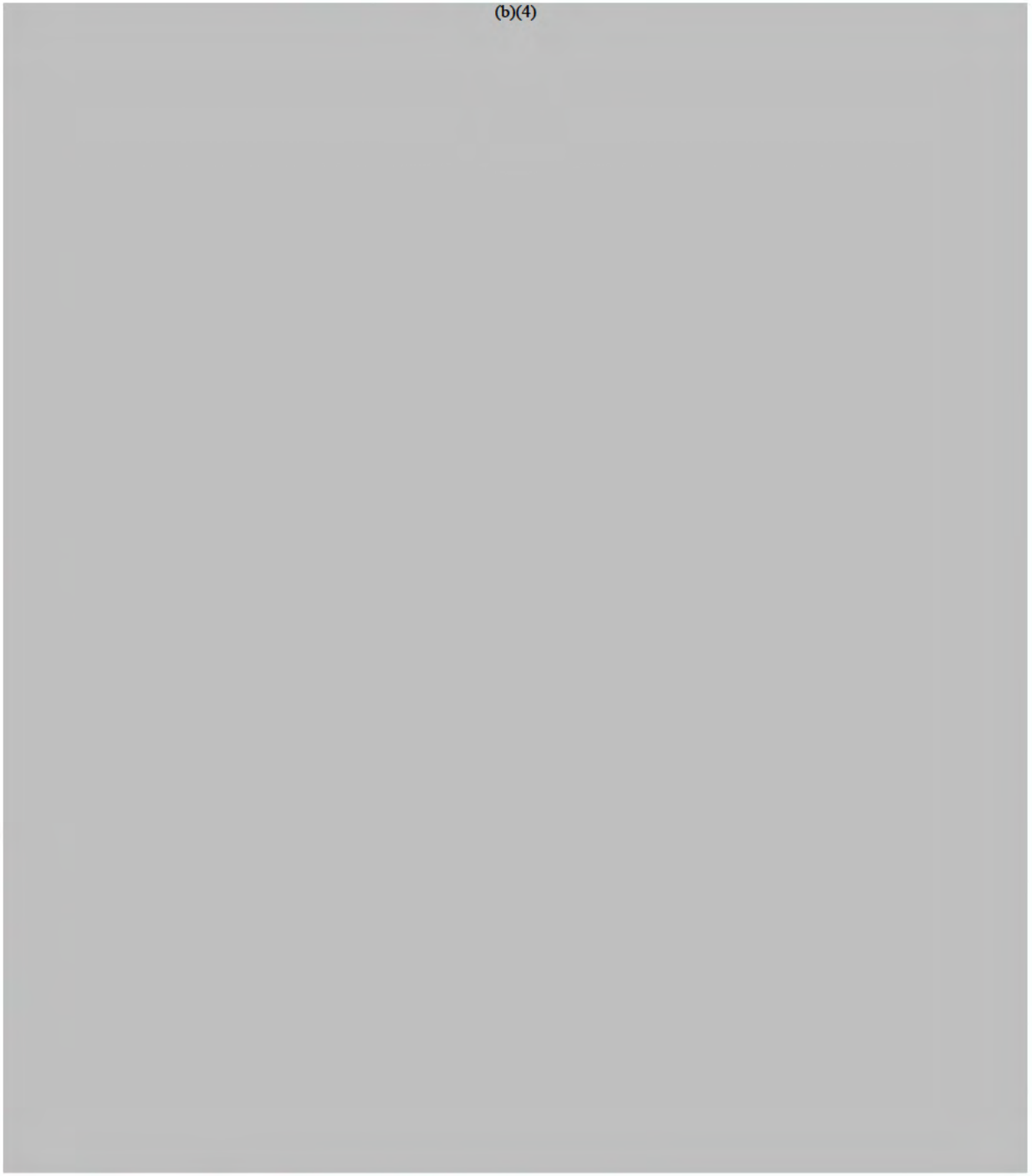

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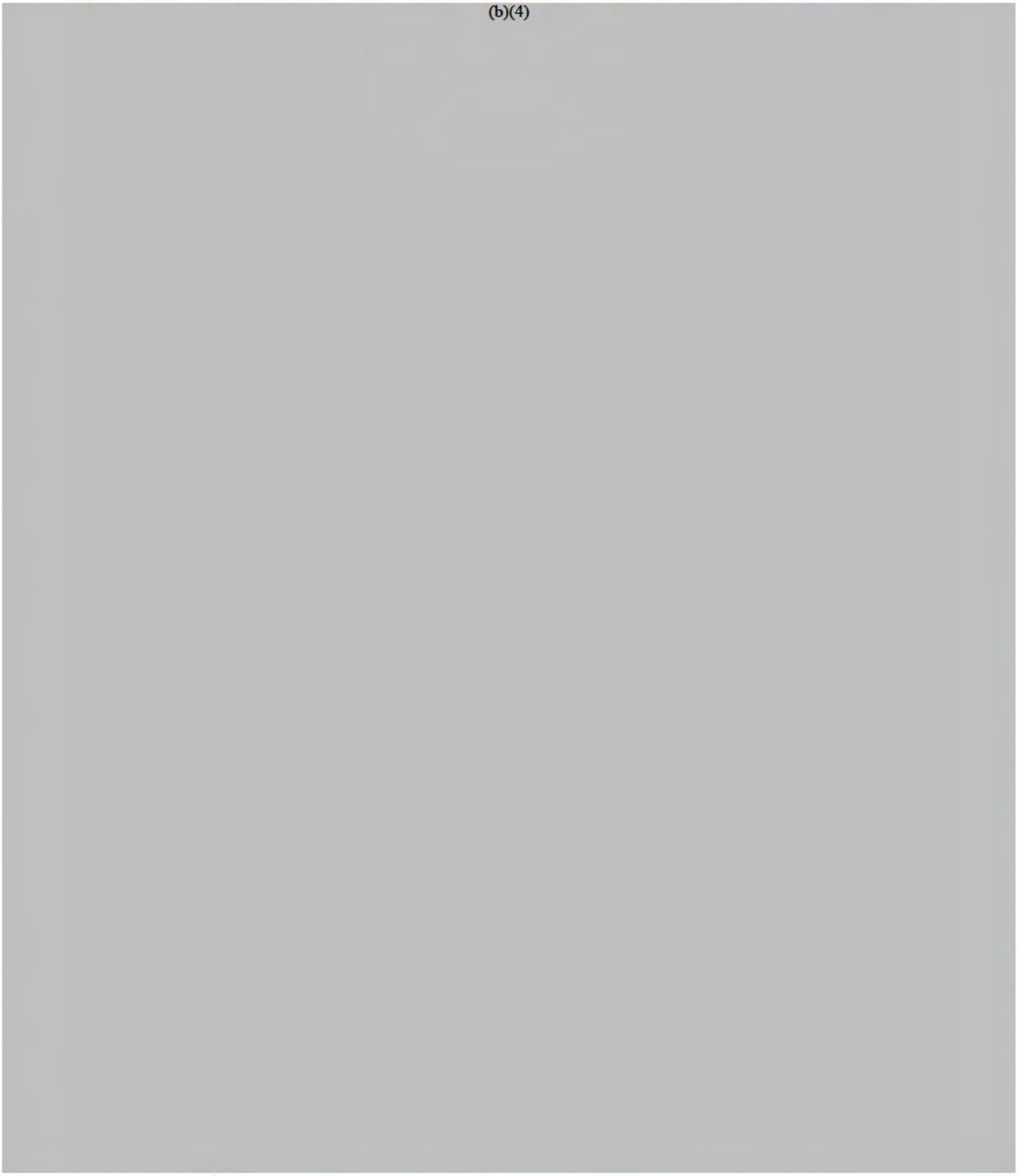

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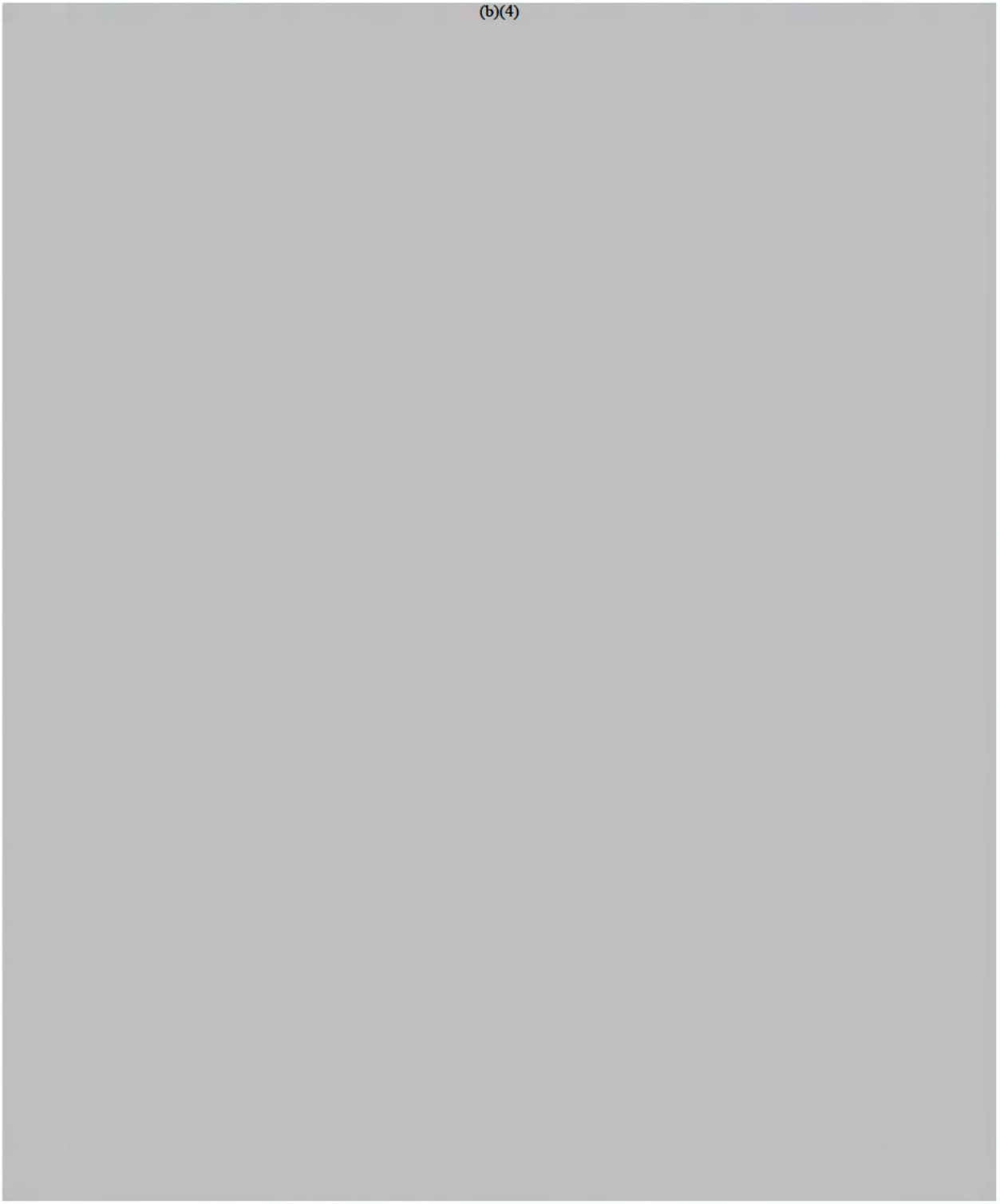

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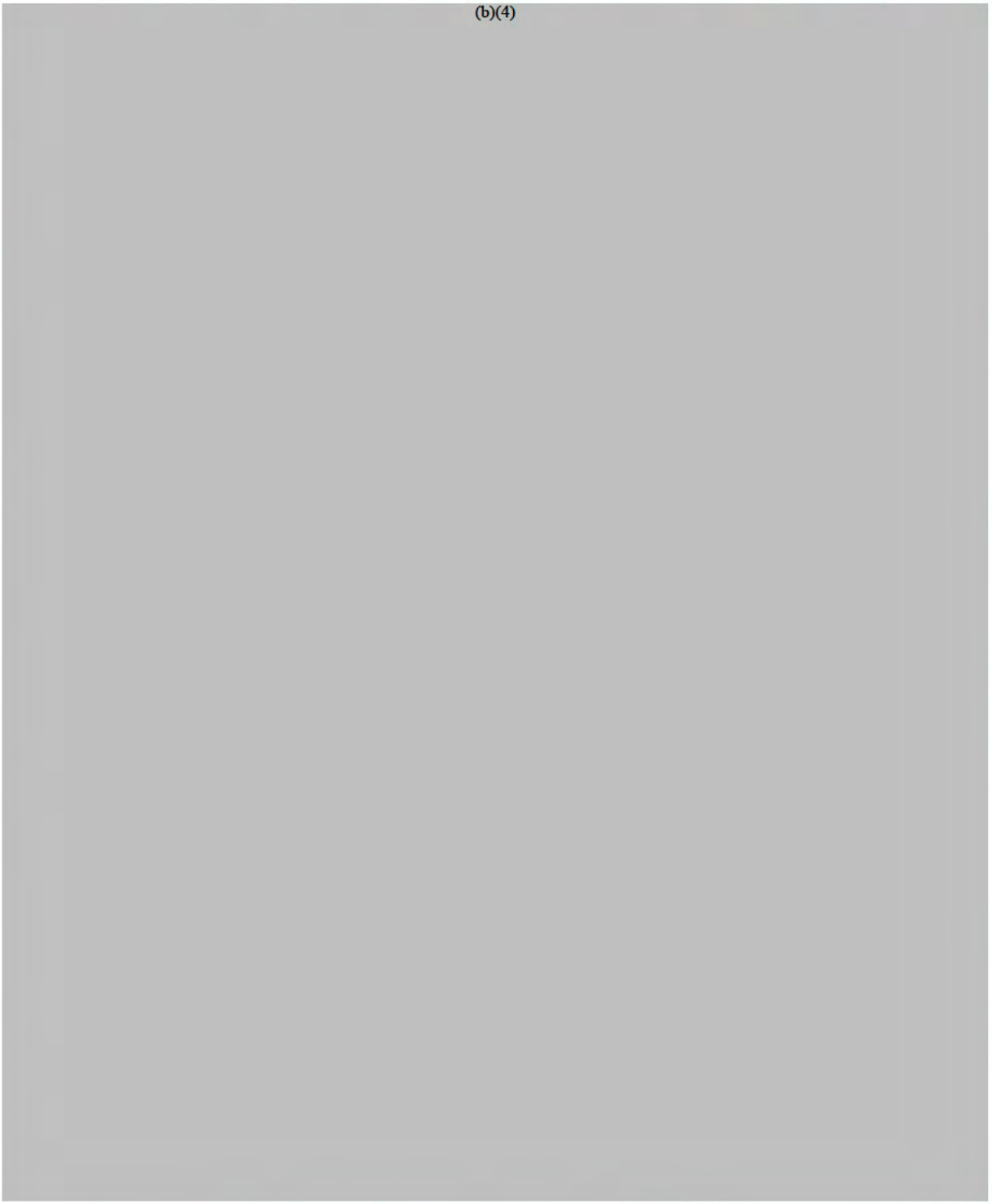

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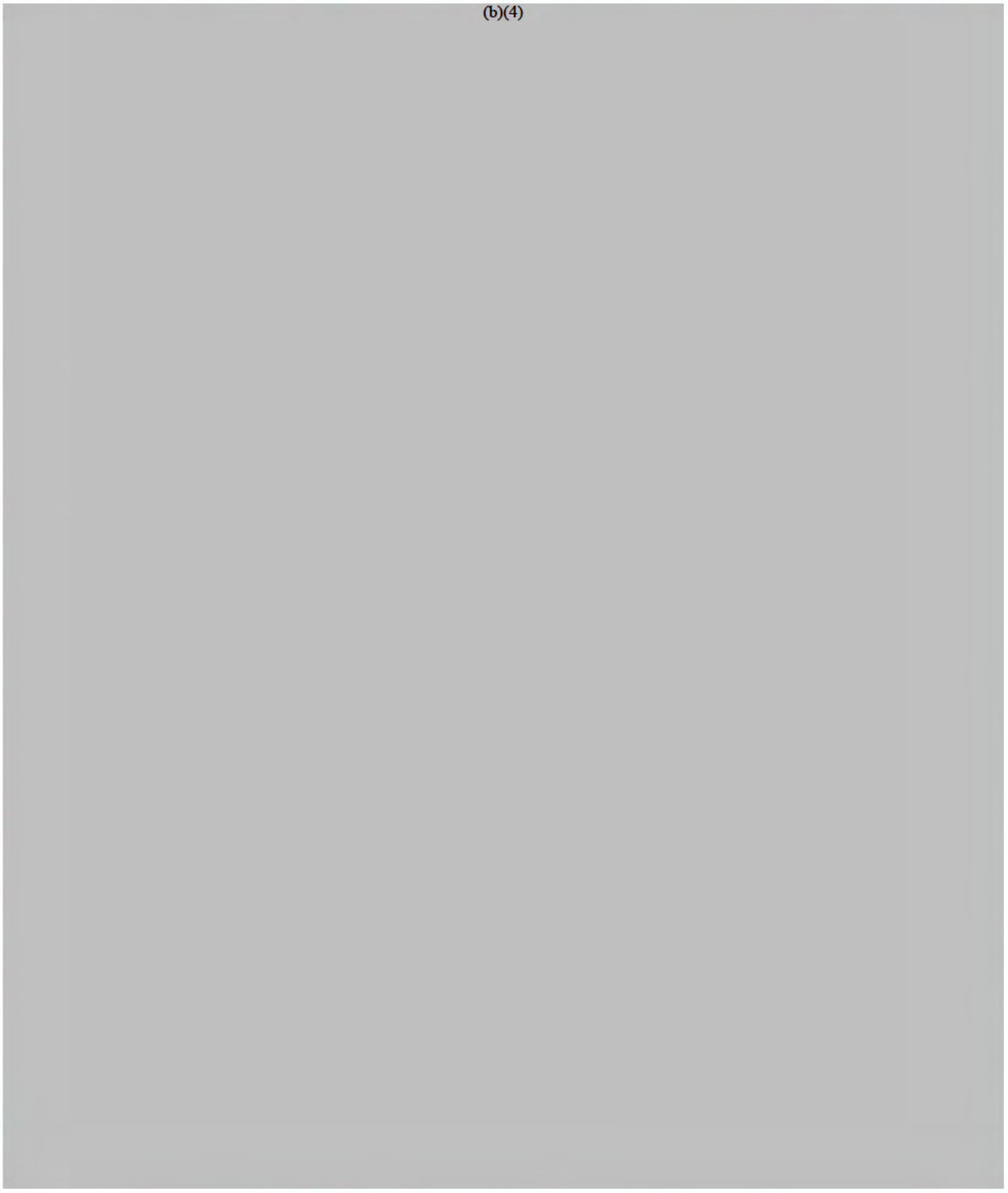

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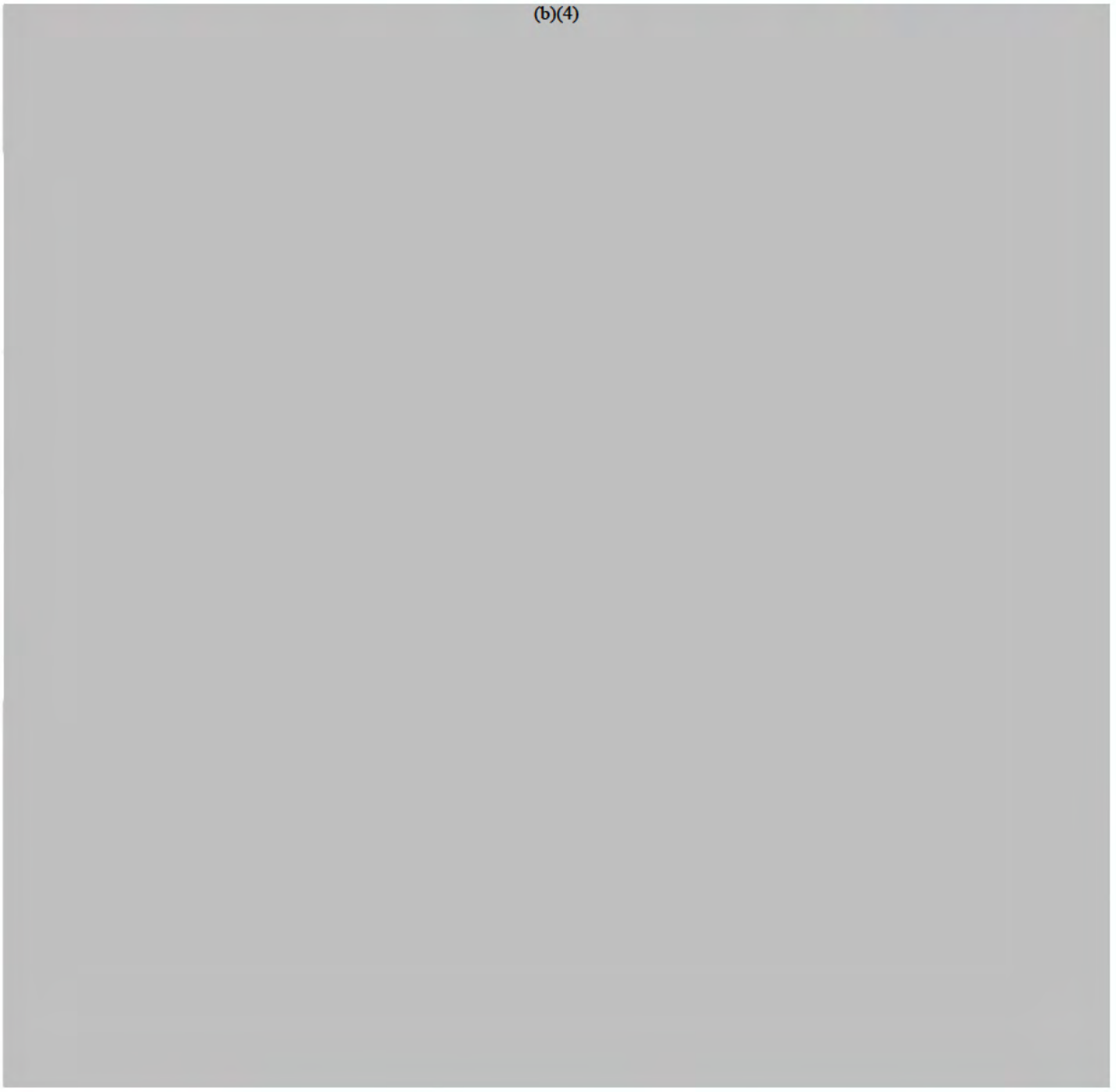

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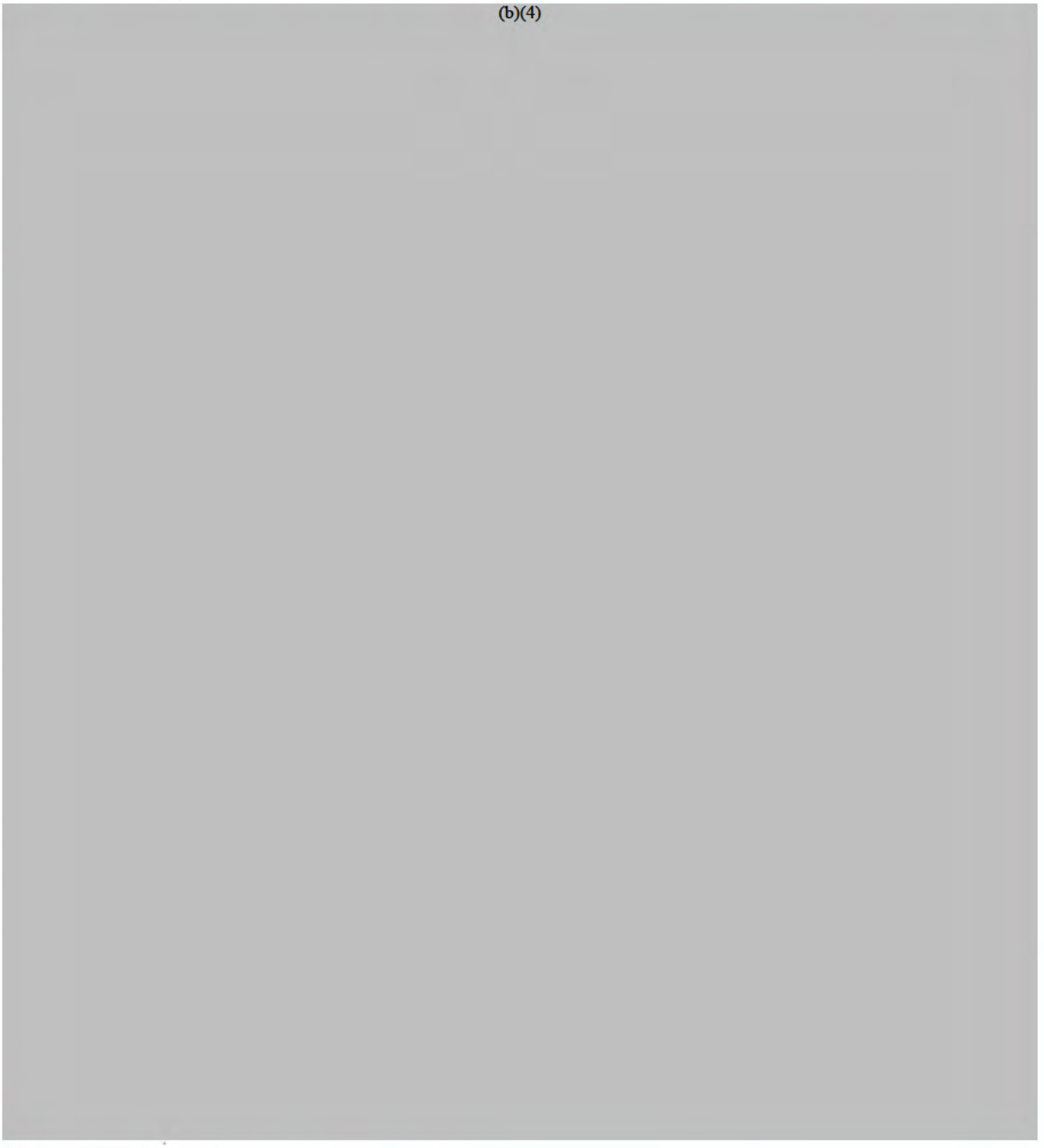

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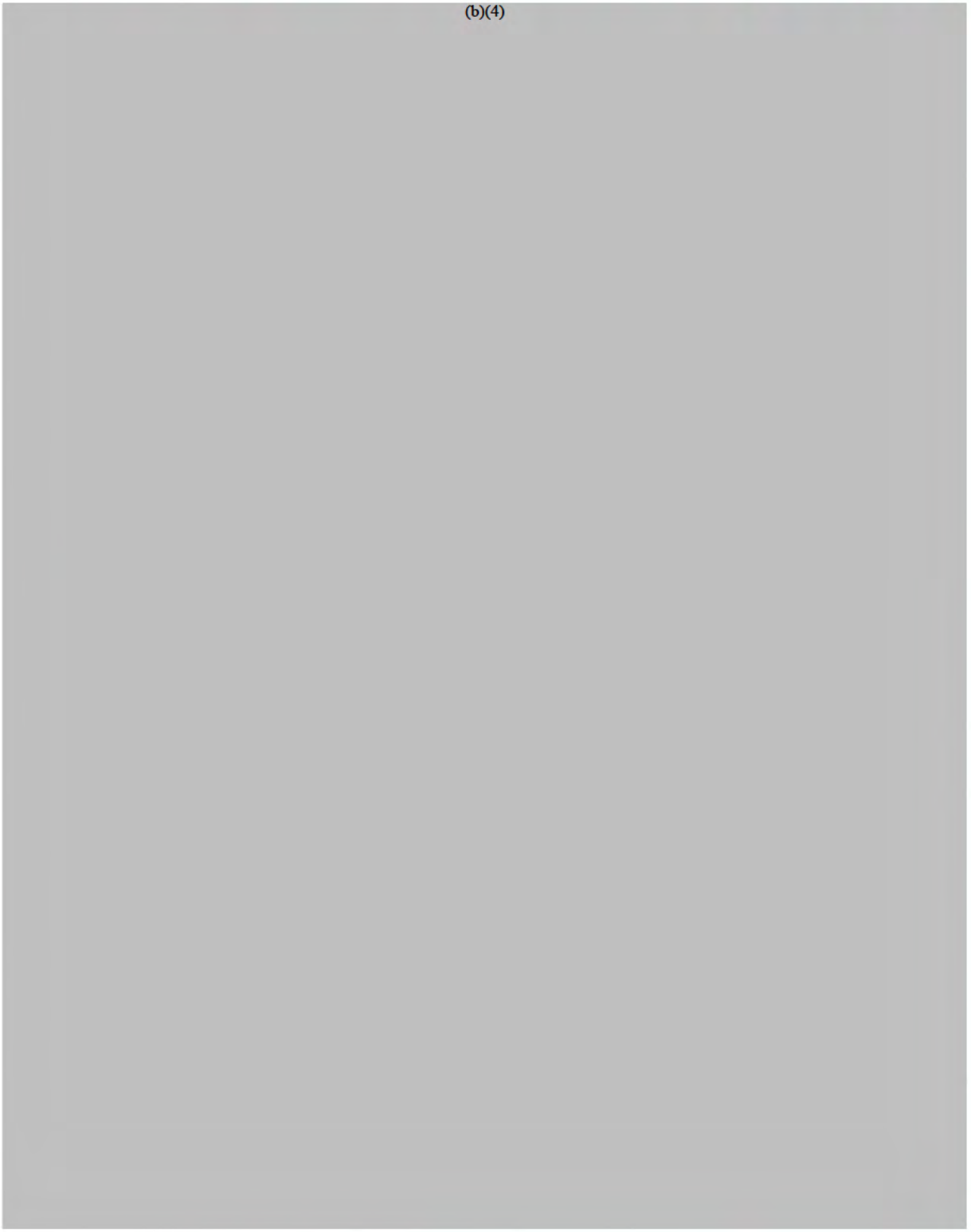
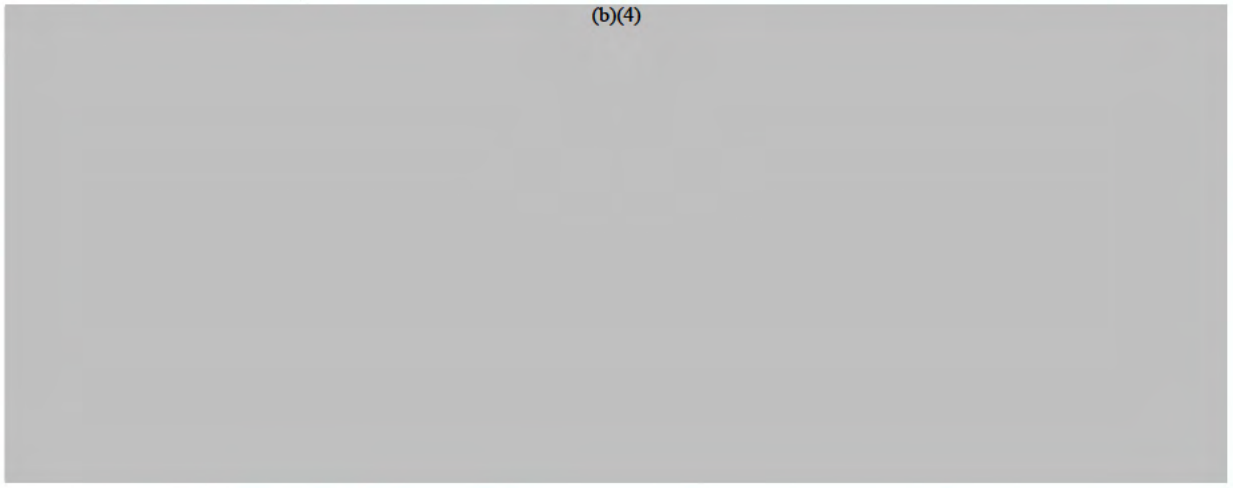
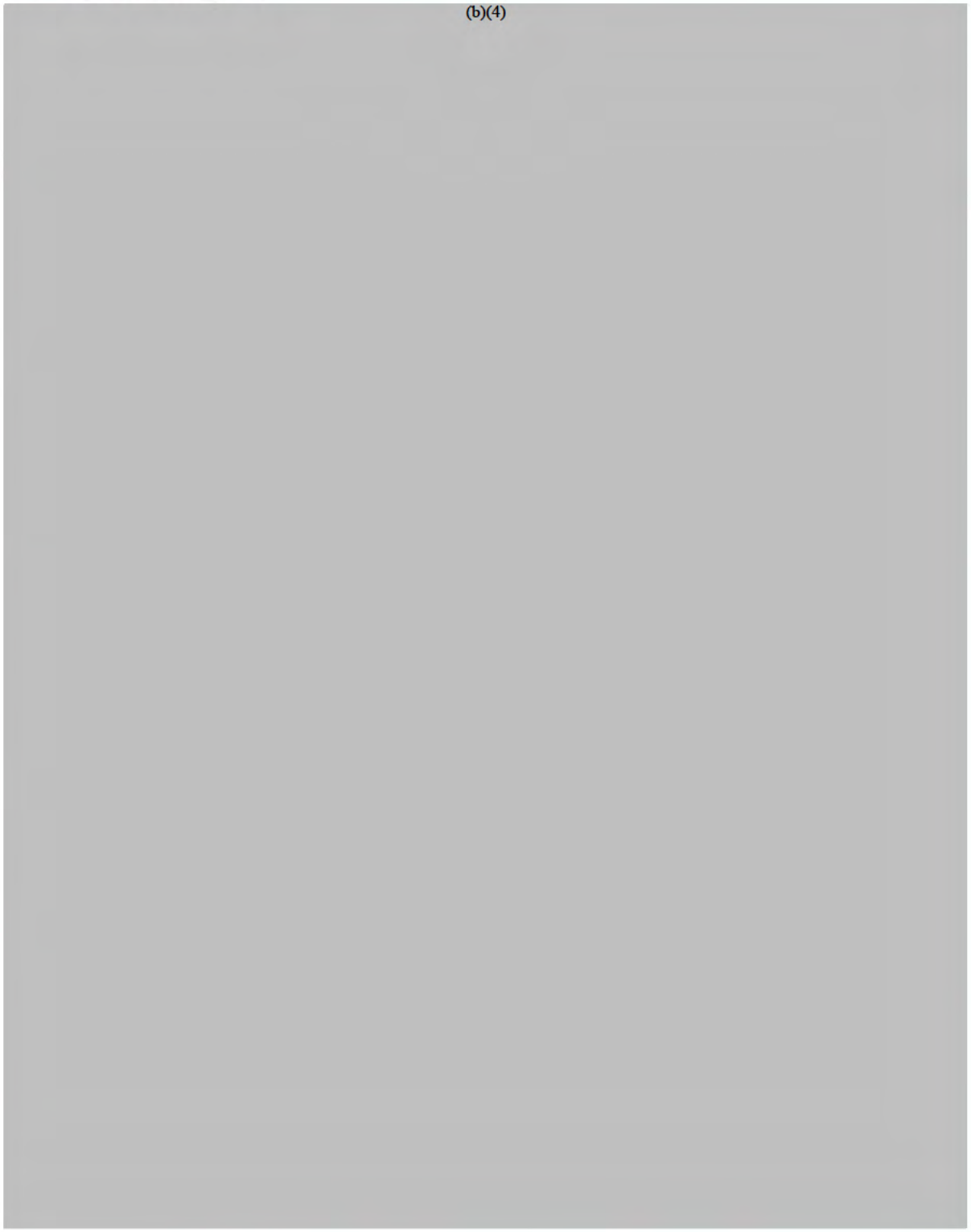


(b)(4)

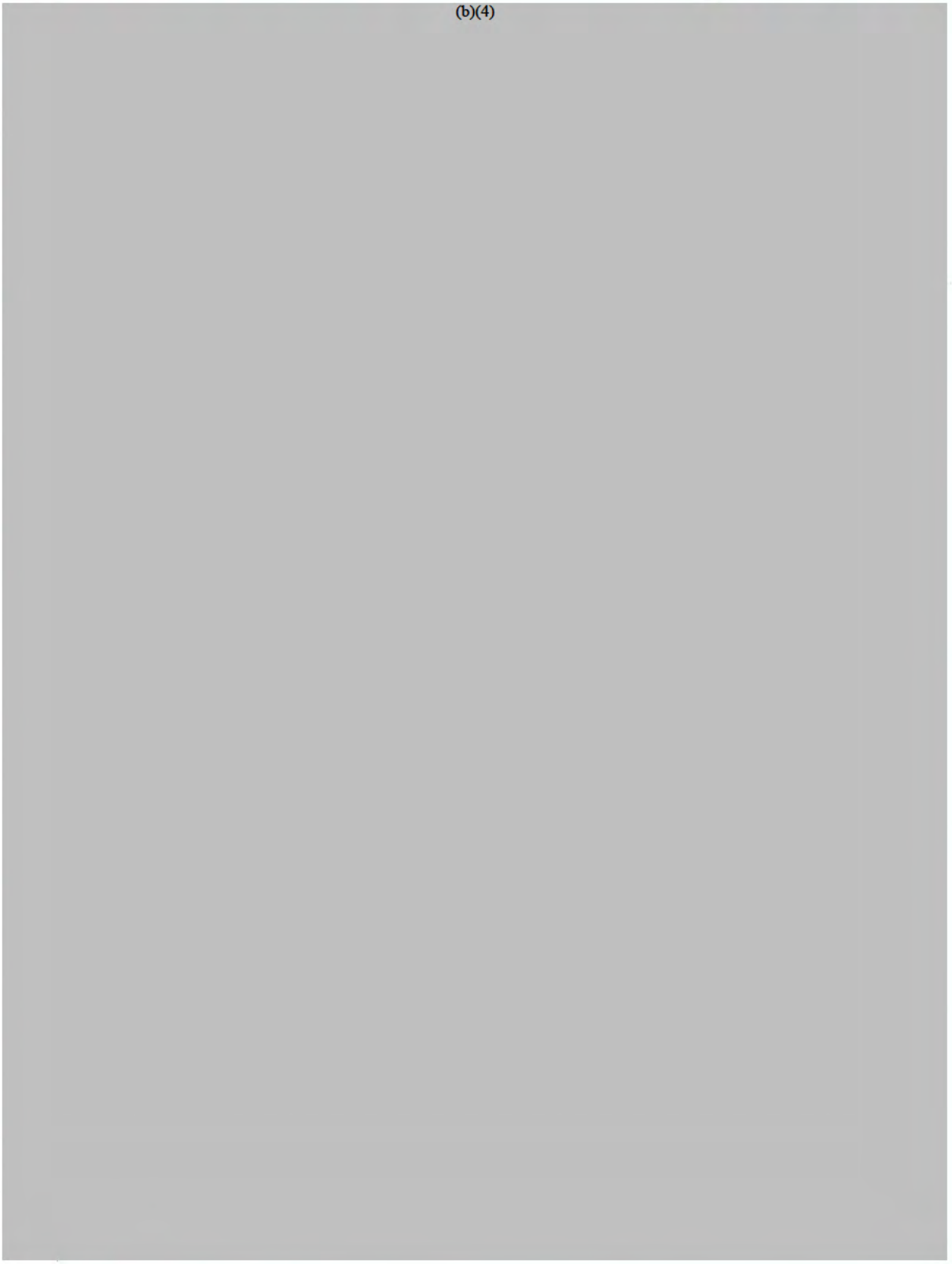


3. Manufacturing

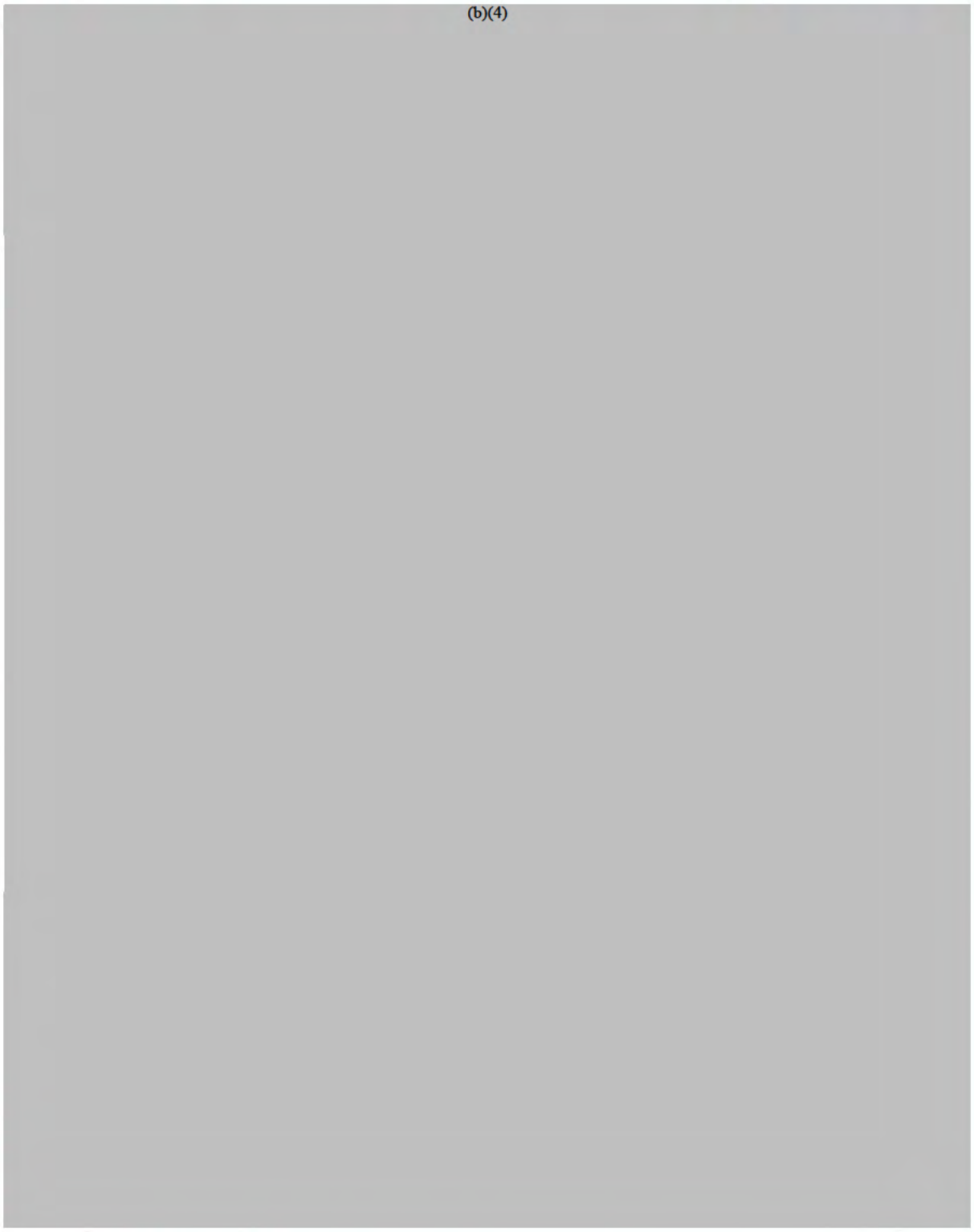
(b)(4)



(b)(4)



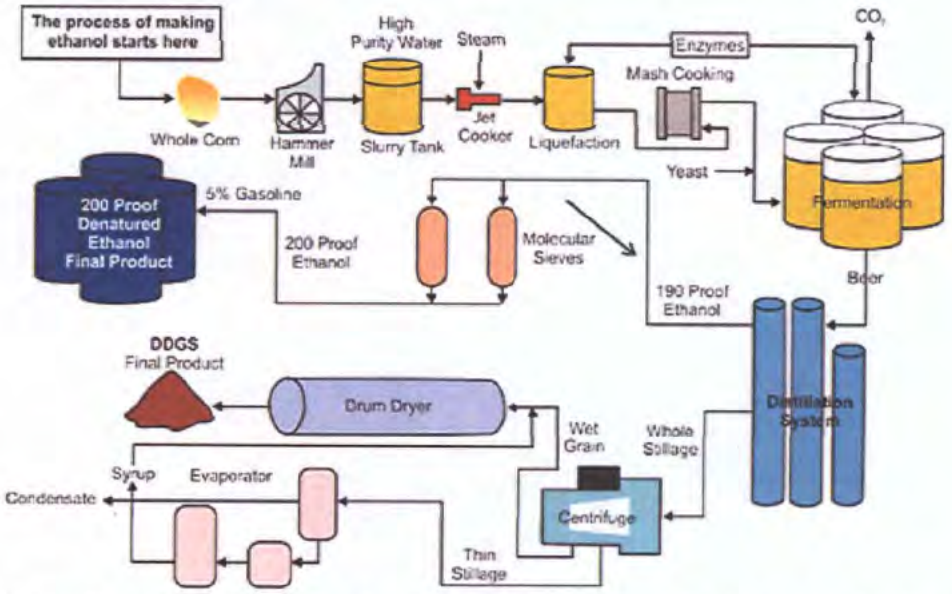
(b)(4)



3.2 Production of the DDG/S containing the Modified *Saccharomyces cerevisiae*

The Modified *Saccharomyces cerevisiae* is designed for the optimal production of bio-ethanol. The inactivated yeast is recovered from the stillage of the bio-fuel plant along with the unfermented plant matter in the same manner as traditional fermentation processes as illustrated in the process flow below.

Figure 2-13 Manufacture of the commercial yeast sold to ethanol plants



(Committee on Water Implications of Biofuels Production in the United States 2008)

3.3 Inactivation of the yeast

Under the current processing methods followed in a fuel ethanol facility, the Modified *Saccharomyces cerevisiae* will be exposed to lethal conditions in the distillation process step ensuring that only non-viable cells are incorporated into the feed of animals. Ethanol boils at 78.4^0 C at normal atmospheric pressure. **Annex 6** contains the results of experiments that confirm that the organism is destroyed at 80^0 C within 5 minutes. The yeast will be in the distillation pot for a much longer time as the entire fermentation batch is distilled prior to transferring the stillage to the recover steps. **Annex 7** contains additional experimental data showing the complete inactivation of the yeast at temperatures below temperatures used in the distillation step. The references Álcohol Textook, 'chapters 16 and 19' (Richards 2009, Earnest, Snyder et al. 2009) provided additional information regarding fermentation plant operations that support the premise that the yeast will be killed during downstream processing at the biofuel plant. On pg 279 in chapter 19 it is noted that '*The maximum temperature recommended for most strains of Saccharomyces cerevisiae is 35°C (at least in a stress-free environment) (Kelsall and Lyons,*

2003). As temperatures climb above 35°C, vital proteins within the cell become structurally altered (denatured), causing a decrease in metabolic activities.'

3.4 The commercial product

3.5 Composition

The Modified *Saccharomyces cerevisiae* is composed of proteins, lipids, carbohydrates, cellulose and minerals. The commercial product for bioethanol production will be sold as either a dry powder produced from a concentrated liquid or the concentrated liquid itself (commercially referred to as 'cream yeast') by DSM Bio-based Products & Services. The inactivated yeast will be a component of Dried Distillers' Grains with or without Solubles (DDG/S) or sold alone as dry yeast.

3.5.1 Specifications

Certificates of analysis for typical Modified *Saccharomyces cerevisiae* batches demonstrate consistency of production lots to specifications (see **Annex 8**).

Table 3-1 Modified *Saccharomyces cerevisiae* liquid concentrate specifications and batch to batch variation

Parameter	Units	Specification (cream yeast)	Pilot Batch Rpt 1202 CRY	Batch C177000	Batch C177001
Appearance		Turbid cream yeast (concentrated broth) with a white/beige color	complies	complies	complies
Dry matter	%	15-20	(b)(4)		
pH		3.8-4.5			
Glucose	g/l	≤ 0.5			
Microbial contamination: total bacterial count	CFU/mL	≤ 10^6			
Microscopic observation in the EOF broth		conform	complies	complies	complies

3.6 Relevant properties

Physical properties of the dry form of Modified *Saccharomyces cerevisiae* are presented in Table 2-18.

Table 2-18 Relevant Physical and Chemical properties of Modified *Saccharomyces cerevisiae*

Properties	Value	Method / Data / Reference
Physical state	Free-flowing granules	Visual
Color	Tan	Visual
Yeast Dry Matter	(b)(4)	Vacuum oven @ 65°C
Moisture		By calculation
(b)(4)		Formulation calculation
	(b)(4)	

3.7 Stability

The Modified *Saccharomyces cerevisiae* has been developed for use in the fermentation of biomass-derived carbohydrates for the purpose of producing fuel ethanol. The organism is not designed for nor is it expected to be stored for long periods of time before use in the biofuel plant. The following information is provided only as an indication of stability as it affects the biofuel processor and not the stability of the inactivated cells present in the Distiller Dried Grains with or without solubles (DDG/S), or dried yeast products that could contain the Inactivated Modified *Saccharomyces cerevisiae* added to the feed of animals.

A pilot plant batch of yeast was split into smaller portions packaged in vacuum sealed, foil lined laminated pouches and stored at two temperatures. Pouches were removed from the storage chambers and an aliquot of yeast was either rehydrated first or added directly to the fermentation broth. Samples were taken from the fermenters at the prescribed times via an auto sampler, the optical density was measured and recorded and this value was used to calculate the cell concentration.

Table 2-19: Biomass in g/L at three fermentation times after storage at 4 and 30 °C

	Storage 4º C				Storage 4º C		
	12 hours	24 hours	36 hours		12 hours	24 hours	36 hours
	rehydrated before use				direct pitch		
Week 0	1,8	3,9	4,1				
Week 7	1,9	3,6	3,7				
Week 9	1,9	3,9	3,1				
Week 17	1,8	3,7	3,9		1,0	3,8	4,0
Week 26	1,0	4,3	4,2		0,2	2,2	4,3
Week 52	nd	nd	nd		0,1	0,1	0,2
	Storage 30º C				Storage 30º C		
	12 hours	24 hours	36 hours		12 hours	24 hours	36 hours
	rehydrated before use				direct pitch		
Week 0	1,8	3,9	4,1				
Week 7	1,7	3,6	3,6				
Week 9							
Week 17					0,3	2,4	4,1
Week 26	0,4	2,8	4,1		1,3	3,9	4,2
Week 52					0,1	0,1	0,1

The data shows that active Modified *Saccharomyces cerevisiae* retains its fermentative capacity after 6 months at 4° C, the recommended storage temperature. Similar results have been found for wine yeast (Simpson & Tracey 1986).

3.8 Nutritional Properties

Two pilot plant batches and a sub-sample of one commercial batch of Inactivated Modified *Saccharomyces cerevisiae* were sent to a third party commercial lab for nutritional analysis. A sample of a commercial baker's yeast was included for comparison because baker's yeast has long been an accepted ingredient in animal feed due to its nutritional properties. In addition, these nutritional analyses were compared to the nutritional data of active dry baker's yeast as published by the USDA.

Complete reports of the performed nutritional analyses are in **Annex 9**

Table 2-20 Nutritional properties of the active Modified *Saccharomyces cerevisiae*

Parameter	Units	Leavening agent, active dry baker's yeast*	Lot 1	Lot 2	Lot3	Baker's yeast (b)(4)
			1315 A	K160 A	1313 EF	
Dry Matter	%	94.9	92.2	92.1	89.7	95.9
Energy	kcal/Kg	3250	3218	3253	3145	3405
Protein	%	40.44	46.10	46.15	46.01	44.09
Fat	%	7.61	6.16	6.09	5.77	4.17
Crude Fiber	%	26.9**	<0.2	<0.2	<0.2	<0.2
Ash	%	NA	9.06	7.85	8.10	4.57
Ca	%	0.03	0.005	<0.004	0.004	0.09
P	%	0.637	1.88	1.54	1.33	0.79
K	%	0.955	2.26	2.54	2.16	1.55
Mg	%	0.054	0.27	0.18	0.19	0.09
Zn	ppm	79	121	106	93	80
Mn	ppm	NA	8	7	11	5
Cu	ppm	NA	7	9	12	2
Fe	ppm	22	49	11	68	54

NA: not available

* USDA National Nutrient Database for Standard Reference Release 26

** Total dietary fiber

The Modified *Saccharomyces cerevisiae* is derived from the same genus and species as baker's yeast. The nutritional composition of Modified *Saccharomyces cerevisiae* is therefore expected to be very comparable to that of dry baker's yeast already on the market. This was confirmed by comparing the nutritional composition of three lots of Modified *Saccharomyces cerevisiae* with the composition of active dry baker's yeast obtained from the USDA National Nutrient Database (USDA 2013), and the commercialized baker's yeast (b)(4) (see Table 2-20 above).

The difference in fiber content is due to methods of expressing fiber. In the active dry baker's yeast reported on the USDA database, the total dietary fiber content was determined, whereas only crude fiber content was measured in Modified *Saccharomyces cerevisiae* and (b)(4) baker's yeast.

Ash content is driven by the media composition. Baker's yeast has been commercially produced for over 75 years and the media composition has been optimized to be the least expensive formulation that allows for maximum cell mass in the shortest period of time. The media formulation for the Modified *Saccharomyces cerevisiae* has not been optimized at this point in

time and may be too rich in minerals. DSM anticipates that the concentration of the media components may be altered in the future.

The difference in protein is driven by differences in the parent strain, media composition and the differences in the fermentation process. The Modified *Saccharomyces cerevisiae* has been developed to efficiently perform in biofuel substrates such as crude corn digests. Baker's and brewer's yeast used in feed are valued for their protein content and an increase in protein would be welcomed by the feed industry.

High protein levels (up to 60% on dm is possible) lead to high yeast activity in bread applications, however they lead to a lower shelf life of the (fresh) yeast. In the case of dry yeast, an additional factor is that high protein yeast (b)(4) .

All in all, the yeast protein in baker's yeast products may vary from 40% to 60-65% on dm. For active dry yeast it may vary from 40-55% on dry matter (Reed, Peppler 1973).

The specification in the 2013 AAFCO OP definition 96.1 for dried yeast (Association of American Feed Control Officials 2013) is that the organism is *Saccharomyces cerevisiae* and has a minimum protein content of 40%. The Modified *Saccharomyces cerevisiae* easily meets this requirement.

The slightly low dry solids content for sample 1313EF presented by the outside laboratory is inconsistent with the sample analysis at the time of manufacture, see Annex 10, were the plant QC department recorded a dry solids of 92.2% which was within specification when the sample was taken for shipment to the analytical laboratory. Yeast is a hydroscopic substance and short exposure to high humidity, such as a rainy day, can quickly affect the total moisture of an open container. We suspect that during shipment or handling at the analytic lab the sample gained moisture.

Yeast is utilized as an animal food ingredient for its nutritional composition, the Modified *Saccharomyces cerevisiae* has a nutritional composition that is substantially similar to traditional yeast utilized in feed. Feed manufacturers use sophisticated nutritional formulation programs to produce batches of feed and adjust each batch based upon the compositional make-up of the ingredients. This allows the feed manufactures to compensate for ingredient availability and seasonal variation in composition. Any difference between the Modified *Saccharomyces cerevisiae* and for example a Baker's yeast in protein level would be easily managed by a feed mill nutritionist.

4. Dried Distillers Grains

The inactivated Modified *Saccharomyces cerevisiae* may become a component of distillers dried grains (DDG) in the same manner as traditional yeast used in grain fermentations and the genetically engineered *Saccharomyces cerevisiae* produced by the Mascoma Corporation that was listed by AAFCO after CVM review. DSM asked a potential customer who produces bio-fuel to evaluate the DSM Modified *Saccharomyces cerevisiae* in their process and to provide detailed analyses of the resultant distillers products. Three batches of Distillers Dried Grains with Solubles (DDGS) containing the Inactivated Modified *Saccharomyces cerevisiae* were produced via fermentation of #2 yellow corn suitable for use for animal feed in 2012 following the commercial bio-fuel producer's current process. Corn is the most common substrate for bio-ethanol production with about 5 billion bushes being utilized for fuel in 2013. (ERS 2014)

Table 4-1 Ingredients used in the bio-fuel fermentation

Input	US Approval
	(b)(4)

Input	US Approval
	(b)(4)

The product data sheets for each ingredient are in Annex 11.

4.1 Comparison of DDGS produced with conventional and Modified *Saccharomyces cerevisiae*

Four samples of DDGS produced with a conventional ethanol production yeast and four with the DSM Modified *Saccharomyces cerevisiae* were produced in a potential customer's plant using #2 yellow corn suitable for use for animal feed as the fermentation substrate. The samples were analyzed in the plant's laboratory using AOCS or AOAC methods as applicable.

Table 4-2 Gross composition

	Method		AOCS Ba 6a-05	AOCS Am 5-04	AOAC 990.03	AOAC 979.10	AOAC 942.05	NFTA 2.2.2.5
Sample	Yeast	Oil Reduced	% Crude Fiber (Dry Basis)	% Fat (Dry Basis)	% Protein (Dry Basis)	%Starch (Dry Basis)	% Ash (Dry Basis)	% Total Solids
503	Modified *Saccharomyces cerevisiae*	No	5.49	9.90	45.11	0.49	4.10	93.68
507	Modified *Saccharomyces cerevisiae*	No	5.45	10.72	41.83	2.27	3.08	93.48

	Method		AOCS Ba 6a-05	AOCS Am 5-04	AOAC 990.03	AOAC 979.10	AOAC 942.05	NFTA 2.2.2.5
Sample	Yeast	Oil Reduced	% Crude Fiber (Dry Basis)	% Fat (Dry Basis)	% Protein (Dry Basis)	%Starch (Dry Basis)	% Ash (Dry Basis)	% Total Solids
598	Modified *Saccharomyces cerevisiae*	No	5.84	10.29	46.08	1.70	3.13	93.90
599	Modified *Saccharomyces cerevisiae*	No	5.74	11.70	42.37	2.34	2.85	92.95
908	Conventional *Saccharomyces cerevisiae*	Yes	Not Tested	9.46	29.91	7.19	5.85	90.81
923	Conventional *Saccharomyces cerevisiae*	Yes	7.92	7.65	29.68	9.86	2.74	92.83
928	Conventional *Saccharomyces cerevisiae*	Yes	Not Tested	9.98	28.56	9.45	7.71	87.47
924	Conventional *Saccharomyces cerevisiae*	Yes	5.32	9.04	29.37	11.6	4.77	89.88

Discussion

The primary difference in the nutrient composition of the DDGS resulting from the use of the Modified *Saccharomyces cerevisiae* is an increase in protein concentration. This is due to the fact the Modified *Saccharomyces cerevisiae* ferments xylose thus reducing the amount of carbohydrate solids in the DDGS. A fermentation using conventional yeast has a higher percentage of carbohydrates because the traditional yeast cannot ferment pentoses such as xylose and arabinose, which can be up to 2.75 percent of the carbohydrates in corn remains in the DDGS. See Berger 2006 and Hromadkova & Ebringerova 1995.

4.1.1 Amino Acids

The primary nutrient provided by yeast is protein. Two factors affect the nutritional value of a protein, the amino acid profile and the digestibility of the protein molecules. A more detailed analysis of the amino acid profile and digestibility were performed by the University of Illinois under the direction of Dr. Carl Parsons.

Annex 12 contains the data tables from the laboratory and the statistical analysis.

Table 4-3 DDG amino acid profile comparison, % of protein (dry basis)

Amino Acid	Average Modified Yeast	Average Conventional Yeast	Std Deviation Modified Yeast	Std Deviation Conventional Yeast	P value
ASP	1.88	1.97	0.106	0.105	0.249
THR	1.10	1.16	0.054	0.049	0.209
SER	1.40	1.33	0.084	0.063	0.277
GLU	4.90	4.20	0.352	0.521	0.067
PRO	2.36	2.12	0.134	0.225	0.122
GLY	1.10	1.27	0.070	0.064	0.011
ALA	2.17	2.10	0.112	0.126	0.457
CYS	0.55	0.54	0.026	0.042	0.714
VAL	1.50	1.61	0.084	0.124	0.196
MET	0.64	0.60	0.039	0.057	0.390
ILE	1.18	1.19	0.058	0.067	0.856
LEU	3.79	3.46	0.182	0.285	0.099
TYR	1.22	1.16	0.070	0.083	0.297
PHE	1.56	1.60	0.081	0.141	0.639
LYS	0.75	0.88	0.077	0.067	0.055
HIS	0.72	0.82	0.056	0.041	0.027
ARG	1.18	1.47	0.101	0.107	0.056
TRP	0.21	0.25	0.031	0.018	0.205

Conclusion

There is no statistically significant difference in the amino acid profile of the DDGS produced using the Modified *Saccharomyces cerevisiae* and the conventional yeast with the exception of glycine and histidine, two non-essential amino acids. Therefore the Modified *Saccharomyces cerevisiae* is substantially similar to the conventional yeast already being used as a feed ingredient.

4.1.2 Digestibility

The same samples that were analyzed for the amino acid profiles were also evaluated for digestibility in roosters.

Rooster feeding trials were performed by Dr. Carl Parsons – University of Illinois. Mature roosters that were cecetomized were used to determine digestibility. Statistics are in Annex 13.

- Adult leghorn cecectomized males were used.
- Roosters were fasted for approximately 27 hours.
- Each sample was fed to 4 roosters and the excreta from each rooster was pooled by weight after drying.
- Roosters were fed approximately 25g.
- Roosters were then placed in cages with ad lib water access with a collection tray underneath for 48 hours.
- Excreta were collected from the trays 48 hours after feeding. Excreta were collected into pre-weighed freezer containers using water.
- Excreta were freeze dried and weighed. Then the excreta were ground using a standard small coffee grinder.
- As pooled samples were used, the pool was weighted so that the rooster with the most contributed the most to the pool.
- The feed and pooled excreta were sent to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories for Amino Acid analysis. AOAC Official Method 982.30 E(a,b,c), chp. 45.3.05, 2006. http://www.aescl.missouri.edu/MethRefs.html
- After results were received, the values were placed into a spreadsheet using an endogenous correction (AA values for fasted rooster excreta) and then the percent of each amino acid that was digested was determined.

Table 4-4 DDGS amino acid digestibility

Amino Acid	Average Modified Yeast	Average Conventional Yeast	Standard Deviation Modified Yeast	Standard Deviation Conventional Yeast	P value
ASP	72.87	69.17	2.23	3.71	0.249
THR	73.26	71.16	2.75	2.46	0.209
SER	79.22	75.53	2.52	5.29	0.277
GLU	84.16	80.75	0.89	3.93	0.066
PRO	80.79	79.68	1.26	3.62	0.122
ALA	84.16	81.39	1.15	2.78	0.0578
CYS	68.62	67.73	1.67	4.93	0.3727
VAL	78.43	77.23	2.26	2.21	0.2370
MET	85.64	82.20	1.18	2.92	0.0357
ILE	79.58	77.95	2.42	1.85	0.1626
LEU	86.99	86.72	1.08	1.54	0.3927
TYR	86.69	82.47	1.75	2.45	0.0156
PHE	84.60	82.74	1.56	1.64	0.0758
LYS	57.11	50.44	5.41	6.59	0.0844
HIS	75.83	75.54	1.68	3.26	0.4398
ARG	84.29	82.30	2.68	2.38	0.1551
TRP	87.97	87.94	1.79	2.90	0.4927

Conclusion

There is no statistically significant difference in digestibility between the Modified *Saccharomyces cerevisiae* and the conventional yeast except for methionine and tyrosine which may be due to three samples of the conventional yeast DDGS having both methionine and tyrosine digestibility well below the fourth sample which in turn was similar to the level of the modified yeast samples. Laboratory technician error may be the cause for the difference.

4.1.3 Metabolizable Energy

Total Metabolizable Energy (TME) was determined by Dr. Carl Parsons – University of Illinois using intact roosters.

- The feed and pooled excreta were sent to the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories for nitrogen (N) analysis. Combustion Analysis (LECO) AOAC Official Method 990.03, 2006. http://www.aescl.missouri.edu/MethRefs.html
- Dry matter (DM) of feed was performed in the Animal Science Laboratory - University of IL. Approximately 2g of feed is placed in a pre-weighed beaker and the total weight is recorded. Feed is dried at 105C for 24 hours, allowed to cool for about 30 minutes, then weighed.
- Gross energy (GE) was performed in the Animal Science Laboratory - University of IL with a Parr 1261 bomb calorimeter. GE was performed on the feed, as-is, and the pooled excreta.
- The GE of feed and excreta, the N of feed and excreta, and the DM of feed were placed into a spreadsheet where endogenous values again were used to determine the TME of the feedstuff.

Table 4-5 Metabolizable Energy

Sample	ID	Oil Reduced	Gross Energy of feed as-is(kcal/g)	Dry Matter Percent	TME_n (kcal/g DM)
503	Modified *Saccharomyces cerevisiae*	No	5.10	91.70	2.89
507	Modified *Saccharomyces cerevisiae*	No	5.14	92.50	3.09
598	Modified *Saccharomyces cerevisiae*	No	5.17	93.10	2.92
599	Modified *Saccharomyces cerevisiae*	No	5.20	94.60	2.96
908	Conventional *Saccharomyces cerevisiae*	Yes	4.75	89.00	2.96

923	Conventional *Saccharomyces cerevisiae*	Yes	4.84	92.80	2.74
928	Conventional *Saccharomyces cerevisiae*	Yes	4.75	89.80	2.65
924	Conventional *Saccharomyces cerevisiae*	Yes	4.65	88.50	3.16

The average TME for Modified *Saccharomyces cerevisiae* DDGS is 2.965 kcal/g DM whereas the average TME for conventional yeast DDGS was 2.878 kcal/g DM. The observed higher for the Modified *Saccharomyces cerevisiae* value was most probably due to the higher level of lipids in the Modified *Saccharomyces cerevisiae* due to a processing constraint in the plant. None the less, the DDGS from the process using the Modified *Saccharomyces cerevisiae* is a suitable source of energy. If the oil is reduced in the commercial scale production, the nutritional value of the product will be communicated to customers who will use one of the feed formulation software packages to make the necessary adjustments to compensate for nutrient variability.

Conclusion

DDGS is a primary source of protein in modern feed formulation. Based upon the minimal difference in amino acid composition and amino acid digestibility, there is no significant difference between the proteins contained in the DDGS from a conventional yeast fermentation and that of Modified *Saccharomyces cerevisiae*. The increased protein concentration in the DDGS from the fermentation of grains with Modified *Saccharomyces cerevisiae* is due to a reduction in carbohydrates, which is the purpose of using the Modified *Saccharomyces cerevisiae;* converting more carbohydrates to ethanol. The total metabolizable energy of the DDGS from an ethanol fermentation using Modified *Saccharomyces cerevisiae* was greater than from conventional yeast fermentation primarily due to an up-steam processing difference that results in less lipids in the DDG from conventional yeast fermentation.

When all the nutritional properties of the DDGS produced from an ethanol fermentation process utilizing Modified *Saccharomyces cerevisiae* are evaluated, particularly when the availability of sophisticated feed formulation software is considered, it is apparent that the DDGS is suitable for use in animal food.

The above information is provided as an example of possible DDGS nutritional properties; the inherent variability in grain composition due to growing conditions at different locations, yearly weather patterns and fermentation practices may impact the nutrient content of DDGS from bioethanol fermentations which is out of the control of the submitter and would not be due to the use of the Modified *Saccharomyces cerevisiae*.

5. Information on any self-limiting levels of use

It is not expected that the amount of Inactivated Modified *Saccharomyces cerevisiae* in DDG/S or feed will be higher than the amount of classical *Saccharomyces cerevisiae* already found in feed or DDG/S, as a byproduct of conventional ethanol distillation. It was estimated that dried yeast represents 20% by weight of the dried distillers' solubles (Liu 2011).

The expected animal intake of DDG/S containing Inactivated Modified *Saccharomyces cerevisiae* is equal or less than the current intake of DDG/S containing classical *Saccharomyces cerevisiae* derived from starch-based biomass. These intakes depend on the nutritional needs of the specific target animals and are summarized in the table below (Table 3-1).
Since the nutritional content of Inactivated Modified *Saccharomyces cerevisiae* is very similar to conventional *Saccharomyces cerevisiae* (see Table 2-20), the intake levels of Inactivated Modified *Saccharomyces cerevisiae* by the target animals will not exceed what is expected from conventional *Saccharomyces cerevisiae*.

The following table illustrates the maximum percentage of modified yeast in the diet based upon the literature for DDG/S in the diet and the quantity of yeast in DDG/S (Liu 2011).

Table 5-1 Intake levels of DDG/S in feed of target animals

Species / Class	DDG/S Maximum % of Diet	Modified *Saccharomyces cerevisiae* Maximum % of Diet
Swine (Shurson, Spiehs et al.)		
Nursery pigs (>15 lbs.)	25	5
Grow-finish pigs	20	4
Developing gilts	20	4
Gestating sows	50	10
Lactating sows	20	4
Boars	50	10
Poultry (National Corn Growers Association 2008)		
Chicken - Broilers	10	2
Chicken - Layers	15	3
Turkeys (grow/finish)	15	3
Bovines (Weiss, Eastridge et al.)		
Beef cattle	25	5
Dairy cows	25	5

5.1 Spill-over Analysis

The primary difference between the engineered strain that is the subject of this GRAS dossier and the wild type *S. cerevisiae* is the ability of the engineered strain to efficiently convert xylose into ethanol. The mechanistic reason for the poor ability of *S. cerevisiae* to utilize xylose was report by Batt et al. in 1986. The xylose metabolic pathway is found in many strains of wild type yeast and the pathway is well understood. (Toivari et al. 2004). The parent strains of the GRAS substance were previously studied on three occasions to ascertain the possible production of unusual metabolic by-products when grown on a synthetic medium designed to mimic a plant carbohydrate hydrolysate. In 2004 two engineered strains were compared to the wild-type parent and the only significant difference in metabolite formation was that of an environmentally induced strain RWB202-AFX which produced xylitol whereas the engineered strain and the parent produced almost none, (Kuyper et al. 2004). Analysis of a strain engineered for improved xylose utilization was found to produce 0.5, 4 and 1.5 times as much glycerol, acetate and lactate respectively than the wild type; when the engineered strain was grown on a glucose, xylose mixture vs. the wild type which was grown on glucose. However, the increases in absolute concentration were quite small, < 10 mM and were not different from another strain produced by environmental induction, (Kuyper et al. 2005a). A similar experiment with another engineered strain showed reduced acid production (Kuyper 2005b). A review article by Kim et al. in 2013 noted that modifications of other strains in different laboratories had similar results when the goal is to increase ethanol production via pentose fermentation (Kim et al. 2013). A study of nine strains of *S. cerevisiae* engineered to ferment xylose via different alterations to the pathway revealed minimal production of xylitol on a synthetic media, yeast nitrogen base with 4% xylose.

At the request of CVM a new study was performed to determine if the results obtained with the parent strains in defined media would be the same with (b)(4) in a fermentation broth indicative of a corn hydrolysate found in bioethanol facilities. To that end, DSM obtained corn hydrolysate from a USA based bioethanol manufacturer and fermented the substrate simultaneously in six paired lab fermenters utilizing a wild type parent strain (b)(4) and a commercial yeast marketed for bioethanol fermentation, Ethanol Red. The corn hydrolysate was used at a concentration that the bioethanol manufacturer indicated was typical of that used in commercial production (see table 5.2 for sugar values in corn hydrolysate as determined by HPLC). The fermented broth was collected after 48 hours as is the common practice in commercial bioethanol manufacture. The broth was filtered to remove the yeast and other suspended solids and analyzed by HPLC with two methods to cover quantification for the same xylose metabolites as done in the previously published studies and for volatile acids as the latter were what CVM indicated were of concern for animal health if the DDG from a fermentation utilizing (b)(4) was fed to livestock (see Table 5.3 for metabolite levels at the sampling point at the end of fermentation [48h]). CVM was unable to provide any other direction regarding possible metabolites of concern for the agency. No attempt was made by DSM to identify other metabolites because as noted in section 2.3 of this dossier, and the literature, the optimization of the xylose metabolic pathway does not lead to the production of other metabolites.

The following tables provide the results of the analysis.

Table 5.2 Corn Hydrolysate Composition g/kg (oligosaccharides were not quantified)

	Total disaccharides	Glucose	Xylose	Arabinose	Glycerol	Furfural	Ethanol	HMF	Xylitol
Corn Hydrolysate (fermentation medium	6.9	23.3	22.4	12.0	1.5	0.5	0.5	0.1	<0.1
LoD (g/kg)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 5.3 Spill-over Analysis Results

Metabolite (g/kg)	Xylitol	Xylulose	Ribulose	Succinic acid	Lactic Acid	Formic Acid	Acetic Acid	Propionic acid	Butanoic acid
LoD (g/kg)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Corn Hydrolysate (fermentation medium) replicate 1	<	<	1,3	<	0,1	0,3	2,4	<	<
Corn Hydrolysate (fermentation medium) replicate 2	<	<	1.4	<	0,2	0,5	1,9	<	
TW2015-8A1.1-EtOH Red	<	<	1.4	<	0,1	0,3	2,4	<	<
TW2015-8A4.1 – EtOH Red	<	<	1.4	<	0,1	0,4	2,3	<	<
TW2015-8A3.1 – (b)(4)	<	<	1.4	<	0,2	0,4	2,2	<	<
TW2015-8A6.1 – (b)(4)	<	<	1.4	<	0,2	0,4	2,2	<	<
TW2015-8A2.1 – (b)(4)	<	<	1.4	<	0,1	0,4	2,2	<	<
TW2015-8A5.1 – (b)(4)	<	<	1.4	<	0,2	0,4	2,2	<	<

In summary, the study did not reveal any significant differences between the engineered yeast, the commercial yeast nor the wild type parent. Because the engineered yeast produces metabolites at the same level as a commercial yeast that is utilized in dozens of bioethanol plants currently and has been for over ten years, there is no increase in risk presented by the engineered yeast for an animal consuming DDG containing the engineered yeast.

6. Detailed summary of the basis for Notifier's GRAS determination

6.1 General safety assessment of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has an extensive history of use in food processing. Also known as Baker's Yeast or Brewer's Yeast, this organism has been used from as early as the Stone Age (about 9000 BC) as leavening for bread and as a fermenter of alcoholic beverages (Tucker, Woods 1995). But it was only between 1857 and 1863 that Louis Pasteur demonstrated the role played by yeasts, as the micro-organism responsible for fermentation. He noted at that time that one type of living cells was responsible for the fermentation of bread, beer, wine, cider corresponding to a population of a microscopic fungus, *Saccharomyces cerevisiae*. He discovered that a number of varieties of *Saccharomyces cerevisiae* exist in nature and are more or less adapted to the different fermentations (Pretorius 2000). *Saccharomyces cerevisiae* is considered Generally Recognized as Safe through its use in the brewing, baking and winemaking industry and is the subject of several GRAS Notices, 120, 175, 239, 260, 284, 350, 353 (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175, CFSAN / Office of Food Additive Safety 2008, GRN 000260, CFSAN / Office of Food Additive Safety 2008, GRN 000239, CFSAN / Office of Food Additive Safety 2009, GRN 000284, CFSAN / Office of Food Additive Safety 2010, GRN 000350, CFSAN / Office of Food Additive Safety 2010, GRN 000353).

Nowadays, the use of yeasts is not limited to the production of bread or fermented beverages. Since the 1930s yeast extract products were developed to enhance the flavor of a variety of products such as soups, sauces and ready-to-eat meals. Moreover, because of their nutritional characteristics (protein, vitamin, mineral and amino acid content), preparations of living cells or yeast cell walls have been commercialized as food supplements. Yeast is recommended for dietary supplementation for patients with diabetes type 2, diarrhea, high cholesterol and fatigue due to its high chromium and vitamin B content (see www.vitacost.com). In addition, the organism is widely used for the production of macromolecular cellular components such as lipids, proteins, enzymes, and vitamins (see www.vitacost.com; Moyad 2007, Moyad 2008).

Saccharomyces cerevisiae is a ubiquitous yeast. It is found on our bodies and in the air that we breathe, and it is naturally present on and in foods that man and animals eat regularly.

In its safety evaluation of carbohydrase from *Saccharomyces cerevisiae*, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered that *Saccharomyces cerevisiae* belongs to the group of micro-organisms traditionally accepted as constituents of foods or normally used in the preparation of foods (Joint FAO/WHO Expert Committee on Food Additives 1971).

In 1997 the US Environmental Protection Agency (EPA) issued their safety assessment of *Saccharomyces cerevisiae* and concluded that "*S. cerevisiae* is an organism which has an extensive history of safe use. Despite considerable use of the organism in research and the presence of *S. cerevisiae* in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the human had a debilitating condition. Tests for the factors associated with the virulence of yeasts (i.e., phospholipases) indicate that this organism is nonpathogenic. The organism has not been shown to produce toxins to humans."

(Environmental Protection Agency,1997) *Saccharomyces cerevisiae* was therefore included as a recipient microorganism at § 725.420 for the tiered exemption.

According to scientific experts, a nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism is defined as "one that is very unlikely to produce disease under ordinary circumstances." (Pariza, Foster 1983).

The European Food Safety Agency (EFSA) concluded that baker's yeast (*Saccharomyces cerevisiae*) fits this definition of nontoxigenicity and nonpathogenicity and that it is also not a major food allergen. EFSA therefore gave baker's yeast a Qualified Presumption of Safety (QPS) status (Opinion of the Scientific Committee, 2007). This means that in Europe, baker's yeast can be safely used in food and feed production and is exempt from the need for further safety assessment.

The FDA considers *Saccharomyces cerevisiae* and several derived products safe for consumption. Indeed, FDA has approved dried yeast as an ingredient for food (21 C.F.R. §172.896), and baker's yeast extract has been affirmed by the FDA as a GRAS flavoring agent and adjuvant (21 C.F.R. §184.1983). The FDA has also approved various yeast-derived products for their use in food. These include Baker's yeast protein (21 C.F.R. §172.325), Yeast-malt sprout extract (21 C.F.R. §172.590) and Baker's yeast glycan (21 C.F.R. §172.898).

In addition, GRAS notifications have been submitted and accepted by FDA with no questions for the use of genetically modified yeasts as starter cultures for wine (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175).

6.2 Safety of Inactivated Modified *Saccharomyces cerevisiae* for Production Animals

Because of the close relationship between humans and domestic animals and the practice of feeding domestic animals waste food products, animals have also been exposed to and consumed yeast for thousands of years. During World War II yeast preparations were recommended by the WHO as a protein substitute in animal nutrition. Presently, *Saccharomyces* supplements for animal nutrition can be found on the market. Yeast, yeast extracts and yeast cell wall fractions have been widely reported as safe and with positive influence on growth performance, meat quality and ileal mucosa development in poultry (Zhang, Lee et al. 2005, Owens, McCracken 2007). In the beef cattle industry, yeast-derived cell wall preparations are successfully used to minimize the toxic effects of high-ergot alkaloid tall fescue straw (Merrill, Bohnert et al. 2007). In pig feed, yeasts from *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are successfully replacing soy meal in order to avoid anti-nutritional factors from this type of meal (Spark, Paschertz et al. 2005). This work underlines the innocuousness of yeast and yeast extracts for animals in the different stages of life and in different species. In Europe, living *Saccharomyces cerevisiae* yeasts or extracts are permitted for use in animal nutrition in any animal species with no restriction on the amounts to be used (Dir 82/471/EEC). In the United States, supplements of *Saccharomyces cerevisiae* for animal nutrition are commercially available for a variety of animals including cat, rabbit, rodents, birds, fish, dogs, goats, sheep, horses, cows and pigs. Amounts vary according to the size of the animals, but there is no limit in the duration

of feeding (e.g. www.vi-cor.com, www.enzion.com). Section 96 of the American Association of Feed Control Officials 2013 Official Publication (Association of American Feed Control Officials 2013) lists nine definitions describing material containing or consisting solely of *Saccharomyces cerevisiae* or one or more of its components as being suitable for animal feed with no maximum levels noted other than good feeding practices. The earliest entry is from 1951 indicating that the state feed officials and CVM, due to their review of AAFCO listed materials, have considered *Saccharomyces cerevisiae* to be a safe and suitable feed ingredient for over 60 years.

6.3 Safety considerations due to the nature of modifications of *Saccharomyces cerevisiae*

The US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420). This exemption is permitted because genetically engineered strains of the species were found to have no adverse effects on man or the environment. They also determined that the introduction of intergeneric material will not increase the potential for adverse effects, provided that the genetic material is limited in size, well characterized, free of certain sequences and poorly mobilizable (Environmental Protection Agency, 1997).

For DSM's Modified *Saccharomyces cerevisiae*:

- the only additional heterologous genetic material was (b)(4) and the coding sequence of the inserted (b)(4) limited in size;
- the additional genetic material is completely characterized as it was made synthetically;
- the additional genetic material by default is free from sequences of concern, and the additional genetic material is poorly metabolizable as it is integrated in the genome of the host and lacks sequences which would allow it to be mobilized;
- no antibiotic resistance genes are present;
- no toxigenic genes were inserted;
- no antibiotic production genes were inserted.

The gene insertion was not random but targeted and the locations are known as illustrated in sections 2.3.5 and 2.4 of this dossier. The entire genome of *Saccharomyces cerevisiae* is known and it is free of toxin producing DNA sequences (see http://www.yeastgenome.org/ for the entire genome). It is not possible to induce the formation of a new attribute utilizing the techniques described in the organism construction sections of the dossier.

The Modified *Saccharomyces cerevisiae* that is the subject of this GRAS Notice is also the subject of the Microbial Commercial Activity Notice J13-0007 which was "dropped from review" by EPA in June 2013.

DSM also assessed the safety of the Inactivated Modified Yeast as it relates to its use in foods according to the decision tree guidelines developed by Pariza and Johnson (2001) for food enzymes and later modified by Pariza and Cook (2010) for animal feed enzymes derived from GE organisms. The test article was shown to be acceptable for feed use. In addition, the entire

organism is boiled in acidic aqueous fermentation broth during the distillation step in biofuel production and destroyed as seen in Figure 2-13. The decision tree is in Annex 14.

6.4 Exposure for target animals

It is anticipated that the Inactivated Modified *Saccharomyces cerevisiae* will be utilized in animal feed either as an individual feed ingredient similar to traditional dried yeast or as a component of Distillers' Dried Grains with or without Solubles (DDG/S). As an individual feed ingredient the material will be added to the feed of poultry, swine, bovines, minor species and pets at levels not to exceed good manufacturing practices.

The table below presents the possible range of use and exposure of Modified *Saccharomyces cerevisiae*. It will be used alone or as a component of an ingredient (Dried Distillers' Grains) in the feed of the following production animals as part of a complete ration.

Table 6-1 Proposed use level and exposure of Production Animals to Modified *Saccharomyces cerevisiae*

Species /Class	Maximum Feed Consumption* (Kgs per day)	Maximum Level of Modified yeast in Feed from Table 3-1	Maximum intake of Modified yeast (Kg per day)	Number of days of consumption	Total exposure Kgs of yeast	Exposure (mg Modified yeast/kg BW/day+)
Broiler Chickens	0.11	2%	0.0022	48	0.106	1.22
Laying Hens	0.1	3%	0.003	365	1.09	1.67
Breeding Hens	0.1	3%	0.003	365	1.09	1.67
Piglet	0.2	5%	0.01	56	0.56	1.10
Sows	1.8	10%	0.18	365++	65.7	1.20
Lactating Sows	5.17	10%	0.517	579++	299.3	3.45
Finisher	4	4%	0.16	126	20.2	1.31
Turkeys	0.2	3%	0.006	140	0.84	0.63
Beef Cattle	10.6	5%	0.53	180	95.4	0.97
Species /Class	Maximum Feed Consumption* (Kgs per day)	Maximum Level of Modified yeast in Feed from Table 3-1	Maximum intake of Modified yeast (Kg per day)	Number of days of consumption	Total exposure Kgs of yeast	Exposure (mg Modified yeast/kg BW/day+)
Dairy Cows	22	5%	1.1	1460	1,606	2.09

(Subcommittee on Feed Intake, Committee on Animal Nutrition, National Research Council 1987)

+ Average weight at slaughter

++ (Stalder, Engblom et al. 2009)

6.5 Information that may appear to be inconsistent with the GRAS determination

An extensive literature research on the safety of *Saccharomyces cerevisiae* has been performed by the European Food Safety Agency (Opinion of the Scientific Committee, 2007). The following is an extract of the EFSA report:

Saccharomyces cerevisiae (also known as "baker's yeast" or "brewer's yeast") is mostly considered to be an occasional digestive commensal. However, since the 1990's, there have been a growing number of reports about its implication as an aetiological agent of invasive infection in "fragile" populations. A particular feature of such infections is their association with a probiotic preparation of S. cerevisiae (subtype S. boulardii) for treatment of various diarrhoeal disorders (see below). The nature of S. cerevisiae (subtype S. boulardii) and its clinical applications are reviewed by Buts and Bernasconi (Buts, Bernasconi 2005).

In one review, 92 cases of Saccharomyces invasive infection were presented (Enache-Angoulvant, Hennequin 2005). *Predisposing factors were similar to those of invasive candidosis, with intravascular and antibiotic therapy being the most frequent. Blood was the most frequent site of isolation (78% or 72 patients). S. cerevisiae (subtype S. boulardii) accounted for 51.3% (47 cases) of fungaemias and was exclusively isolated from blood. Special caution should be taken regarding the use of S. cerevisiae (subtype S. boulardii) preparations* (Fleet & Balia 2006). *There are number of recent reports and reviews regarding the safety of S. cerevisiae (subtype S. boulardii) preparations involved in:*

- *A case of Saccharomyces cerevisiae acquired fungaemia* (Cassone et al. 2003, Graf &Gavazzi 2007). *The authors concluded that probiotics should be used cautiously in certain high-risk populations.*

- *A review of the current literature reinforces the view that fungaemia and sepsis are rare complications of the administration of S. cerevisiae (subtype S. boulardii) in immunocompromised patients but confirms that the most important risk factor for S. cerevisiae fungaemia is the use of probiotics* (Herbrecht, Nivoix 2005, Munoz, Bouza et al. 2005). *This raises the question of the risk-benefit ratio of these agents in critically ill or immunocompromised patients who are likely to develop an infection after exposure to high amounts of a microorganism with a low virulence.*

The body of knowledge is considered as sufficient (long history of safe use) with only 92 cases of pathogenic cases involving S. cerevisiae reported in total (15 cases diagnosed before 1990); all patients had at least one condition facilitating the opportunistic development of S. cerevisiae. S. bayanus and S. pastorianus are used in wine and beer production. There are no foodborne infection issues for these species.

On the basis of their assessment, EFSA classified *Saccharomyces cerevisiae* as a QPS (Qualified Presumption as Safe) organism, a non-pathogen and not a major food allergen. *Saccharomyces* has been an approved component of feed since at least 1951 and there are no literature reports that identify *Saccharomyces cerevisiae* as toxic for animals. As stated earlier, this means that it can be safely used in food and feed without additional safety testing.

In addition, a comprehensive literature search was performed on April 15, 2013 by DSM Bio-based Products and Services. The scientific databases Medline, BIOSIS and EMBASE as provided by the host STN were searched simultaneously (see Annex 15). Usual terminology

related to pathogenicity, toxicology or safety was used, and the following keywords were searched: *Saccharomyces cerevisiae*/Baker's yeast/Brewer's yeast,pathogen/tox/safe/allergen.

The search confirmed that although the cases of human infection with *Saccharomyces cerevisiae* are rare, some isolates have pathogenic potential. Some clinical isolates are notably known to be virulent in humans. In addition, several reports evaluating the safety of commercial *Saccharomyces cerevisiae* strains have clearly identified the subtype *boulardii* as a potential pathogen, in particular towards immunocompromised patients. See Cassone et. al., 2003 and Graf & Gavazzi, 2007)

Although very few scientific reports are available on potential adverse effects of *Saccharomyces cerevisiae* for animals, the few toxicity studies that were performed on rats, mice or monkeys with the yeast or with the yeast biomass do not suggest that these animals display a stronger sensitivity towards *Saccharomyces cerevisiae* than humans. (Caballero-Cordoba G.M. and Sgarbieri V.C. (2000), Byron J.K. et al., (1995), Maejima K. et al., (1980).

Recent efforts have focused on attempting to characterize the virulence traits related to *Saccharomyces cerevisiae,* thus enabling the means to better identify the potential pathogenic strains (de Lanos R. et al.,(2011) (McCusker J.H. et al., (1994)). The phenotypic traits of virulence of pathogenic strains are believed to be due to their ability to grow at high temperature (38-42^{0}C), their adherence and their ability to invade host cells, and their ability to produce and secrete degradative enzymes (i.e. proteinase and phospholipase) (de Lanos R. et al., (2011)). This search did not identify any reports of isolates of *Saccharomyces cerevisiae* able to produce toxins against animals or humans, although several studies have shown the ability of the yeast to produce toxins against other yeasts (the so-called killer toxins) (Orentaite et. al. (2012), Soares G.A.M and Sato H.H. (1999)).

In conclusion, we believe that this literature search does not alter the conclusions previously made by several bodies of experts, EFSA, EPA and FDA through their acceptance of several GRAS Notices that *Saccharomyces cerevisiae* can be considered as a safe microorganism and a non-pathogen, although some of the yeast isolates should be regarded as opportunistic pathogens of low virulence.

6.5.1 Methyl Glyoxal

In metabolically active yeast cells, methylglyoxal may be formed during normal cell metabolism. Methylglyoxal synthase catalyzes the reaction from dihydroxyacetonephosphate to methylglyoxal. In 1997 Hashimoto et. al. found that genetically engineered *Saccharmoycese cerevisiae* strain DKD-5D-H produced and increased amount of methylglyoxal, a toxic 2-oxoaldehyde, compared to the wild-type yeast cells. This increase was reportedly due to the gentic manipulations involving the enzymes phosphoglucose isomerase, phosphofructokinase and triosephosphate isomerase.

In construct 1, overexpression of the *GLO1*-gene is brought about. To this end, the *Saccharomyces cerevisiae GLO1*-gene is expressed under control of the PGK1-promoter.

The *GLO1*-gene encodes monomeric glyoxalase I, which catalyzes the detoxification of methylglyoxal (a by-product of glycolysis) via condensation with glutathione to produce S-D-lactoylglutathione (see figure 4-5 below).

Figure 4-5 Methylglyoxal detoxification

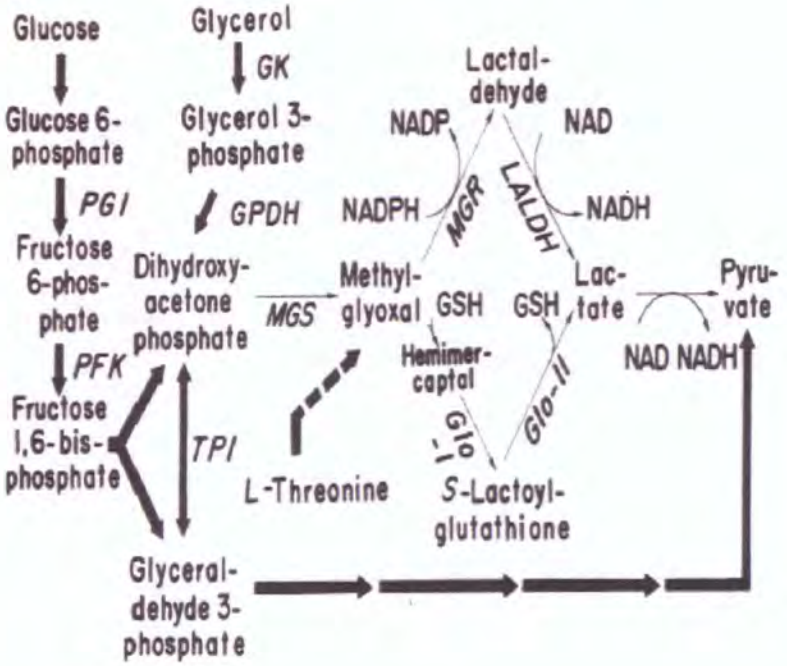


Figure 1 Enzymic routes for synthesis and degradation of MG in *S. cerevisiae*. Non-enzymic formation of methylglyoxal (MG) from glycerol 3-phosphate and glyceraldehyde 3-phosphate is shown by Phillips & Thornalley (1993). The pathway indicated by thin lines represents pathways for MG degradation (glycolytic bypass). For abbreviations, see text.

The figure was copied from Hashimoto et. al. (1997) International Journal of Food Science and Technology **32**, 521-526.

In section 2.5.2 it is noted that overexpression of the GLO1-gene helps in lowering the intracellular glyoxal levels.

6.6 Summary

Saccharomyces cerevisiae is ubiquitous, with man and animals being exposed to the organism on a regular basis through exposure to air, water, soil and food. The organism is composed of primarily proteins, nucleotides, and complex carbohydrates and has not been reported to be toxic nor allergenic for animals. Yeast is utilized as a component of animal food for its nutritional composition, the Modified *Saccharomyces cerevisiae* that is the subject of this notice is not compositionally different from traditional yeast fed to animals as noted in section 2.20 of this dossier. A detailed analysis of the amino acid profile did not reveal any statistically significant differences between the DSM Modified *Saccharomyces cerevisiae* and traditional yeast used for commercial ethanol production from corn.

Saccharomyces cerevisiae has been widely and safely fed to poultry, cattle and other livestock due to its nutritious properties for over 60 years as evidenced by the ingredient definitions in the AAFCO Official Publication. A variety of *Saccharomyces* ingredients for animal nutrition can be found on the market. Yeast, yeast extracts and yeast cell wall fractions have been widely reported as safe and with positive influence on growth performance, meat quality and ileal mucosa development in poultry (Zhang et al., 2005; Owens and McCracken, 2007).

The safety of *Saccharomyces cerevisiae* is recognized by FDA, EPA and EFSA; and is confirmed by the literature. FDA considers *Saccharomyces cerevisiae* and several derived products as GRAS and therefore safe for consumption.

Although there have been reports of infection by *Saccharomyces cerevisiae* in immune-compromised individuals, it is not considered to be pathogenic.

The entire genome of the organism is known and is devoid of known toxigenic sequences. The organism that is the subject of this GRAS Notice was modified following NIH guidelines and has been accepted by the EPA as safe for large scale industrial fermentation and use for the production of bioethanol. The modifications to the organism only increased the number of native genes already present and added one gene for the production of an enzyme that is currently approved for use in food production for both animals and humans. The ability to produce novel amino acids or proteins was not introduced. The gene coding for the non-native enzyme was synthetic and therefore devoid of any extraneous DNA from the template organism. Therefore the Modified *Saccharomyces cerevisiae* does not possess unusual genetic material nor the ability to produce unusual toxic substances that would make its toxicological properties different than that of traditional yeast fed to animals.

It is anticipated that the exposure of target animals to the Genetically Engineered, Inactivated Modified *Saccharomyces cerevisiae* will not be higher than the current exposure to classical *Saccharomyces cerevisiae*. Yeast is already a component of DDG with or without solubles and the DSM yeast is a one for one replacement of the currently used microorganism.

In August, 2013, at their annual meeting, AAFCO accepted the petition of the Mascoma Corporation and formed a new ingredient definition for a genetically engineered *Saccharomyces cerevisiae* developed for bioethanol production from corn that contained four genes from two donor species, a yeast and a bacterium. In their review, CVM noted that the purpose of the 'yeast is to produce fuel-ethanol from dry grind corn and that 'The distillers products will be used in animal food, but will not contain viable bioengineered yeast.'(see CVM, 2013). Therefore, CVM has previously reviewed a genetically engineered yeast used for bioethanol production from a similar substrate and found that the inactivated yeast was suitable for use in a similar feed ingredient, distillers' grains.

Conclusion:

The genetically engineered pentose fermenting *Saccharomyces cerevisae*, refered to in this document as Modified *Saccharomyces cerevisiae*, produced during bio-fuel production from food grade plant- based materials such as corn, wheat and sorghum is safe for consumption by production animals, minor species and pets. The Modified *Saccharomyces cerevisiae* is substantially equivalent to the traditional yeast utilized as an ingredient in animal food.

7. Human safety

Human exposure is anticipated to be limited to individuals handling the material during packaging at the yeast production facility, the bio-ethanol facility, in the manufacture of feed, on the farm, and from consumption of the target animals. The use of personal protective equipment and compliance with normal occupational safety practices will minimize exposure.

To our knowledge, there are no toxicokinetic studies on *Saccharomyces cerevisiae*. However, inactivated Modified *Saccharomyces cerevisiae* will be metabolized during animal digestion into essential compounds consisting mainly of proteins and carbohydrates. Amino acids and peptides are important building blocks in the human body. Carbohydrates are indispensable for storage or transport of energy, and they also play a role in immune function, blood clotting and development.

Saccharomyces cerevisiae is in general considered to be of very low toxicity for humans. This is based on human evidence coming from a long history of safe use, on its composition and on the fact that it is readily biodegradable in the human gastro-intestinal tract. In the public domain, toxicity studies on *Saccharomyces cerevisiae* are limited. However, due to low risk expected for man and the environment, the US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism is exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420).

7.1 Allergenicity

It is well known that when inhaled, proteins in general can cause sensitization and allergic reactions. Inhalation allergy towards *Saccharomyces cerevisiae* has been described by Baldo and Baker (Baldo, Baker 1988). This type of allergy is predominantly observed in bakers.

Regarding oral allergy from ingestion of *Saccharomyces cerevisiae,* only one possible case is known (Pajno, Passalacqua et al. 2005). An atopic 6-year old boy experienced generalized urticaria and asthma after eating pizza and bread fresh from the oven (within one hour). The reaction towards yeast was confirmed in a skin prick test procedure. The reaction only occurred when the bread just had been prepared, whilst no symptoms occurred when the bread was eaten more than one hour after preparation. Since this phenomenon is uncommon with regard to allergens, the cause of the boy's allergic reaction remains inconclusive. In its assessment of *Saccharomyces cerevisiae* for a classification as QPS (Qualified Presumption as Safe) organism, the European Food Safety Agency (EFSA) concluded that *Saccharomyces cerevisiae* is not a major food allergen (Opinion of the Scientific Committee, 2007).

In addition, the impact of the modifications made on the *Saccharomyces cerevisiae* portion of DDGS was evaluated by applying the guidelines on testing the potential allergenicity of genetically modified foods as presented by the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (Food and Agriculture Organization of the United Nations/World Health Organization 2001, Food and Agriculture Organization of the United Nations/World Health Organization 2009). According to the guidelines cross-reactivity between the expressed protein and a known allergen has to be considered when there is:

1) more than 35% identity in the amino acids sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty

or

2) identity of short contiguous amino acids segments (i.e. at least 8 contiguous amino acids). Although the 2001 WHO/FAO consultation suggested searching for matches of 6 identical amino acid segments or longer, it is recognized that a search for such small sequences would lead to the identification of too many false positives (Food and Agriculture Organization of the United Nations/World Health Organization 2009). It has been reported that an immunologically significant sequence similarity requires a match of at least 8 contiguous identical residues (Fuchs, Astwood 1996, Metcalfe, Astwood et al. 1996).

The Inactivated Modified *Saccharomyces cerevisiae* has been engineered to (b)(4) several *Saccharomyces cerevisiae* genes as well as (b)(4) from another yeast species, but no foreign DNA sequences known to code for allergenic proteins were inserted into the organism.

Because (b)(4) not native to *S. cerevisiae*, and *S. cerevisiae* is of low allergenic impact for humans only the foreign gene was extensively evaluated.

For comparison, DSM used the database AllergenOnline™ (available at http://www.allergenonline.org/, last updated February 12, 2013). The comparison was done in March 2013. AllergenOnline™ allows the search in NCBI, SwissProt, PIR, PRF, PDB and the WHO-IUIS databases using a FASTA algorithm. The WHO-IUIS list is set up by the IUIS Allergen Nomenclature Sub-committee operating under the auspices of the International Union of Immunological Societies and the World Health Organization. The objectives of the IUIS Allergen Nomenclature Sub-committee are to maintain a unique and unambiguous nomenclature for allergen molecules and maintain the 'official list of allergens'.

In addition, the amino acid sequence comparison of the inserted heterologous gene protein product (see Annex 16) did not show 35% or more overlap with known allergens using a window of 80 amino acids. Exact matches of 8 amino acids or more were not observed.

Therefore, it is concluded that the inserted gene protein product has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

A material safety datasheet with additional information on the Modified *Saccharomyces cerevisiae* is provided in Annex 17.

8. Environmental Safety

Saccharomyces cerevisae is exempted from TSCA Section 5 PreMarket Notification by the Environmental Protection Agency (40 CFR §725.420, the Environmental Protection Agency 1997). The EPA has reviewed and approved the use of this genetically engineered *Saccharomyces cerevisiae* for biofuel production under Microbial Commercial Activity Notice (MCAN) J11-0001. Another genetically engineered yeast, *Pichia pastoris*, has also been approved under MCAN J04-0003. DSM has filed a Tier 1 exemption notice with the EPA which was accepted by the agency in December 2011 and a Microbial Commercial Acitvity Notice which was accepted on 18 March 2013 and dropped from review on 12 June 2013.

Inactivated Modified *Saccharomyces cerevisiae* is a GRAS substance and per 21 CFR §25.32 (k), foods, food additives and color additives, including GRAS substances, are categorically excluded from the requirement to provide an environmental impact statement or an environmental assesment. Inactivated Modified *Saccharomyces cerevisiae* will be added directly to animal food and is intended to remain in the food through ingestion by animals and it is not intended to replace macronutrients in animal food.

There is no information on the ecotoxicity of Modified *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* in the public domain. However, because of the macro-composition of Modified *Saccharomyces cerevisiae*, consisting mainly of proteins and carbohydrates, it can be expected that the substance is readily biodegradable. Thefore, no toxicity for invertebrates, aquatic plants and fish is expected, since the substance will be degraded by bacteria in the water. In addition, as was previously indicated, *Saccharomyces cerevisiae* has been fed widely to poultry, cattle and other livestock due to its nutritious properties and is thus of very low toxicity. It is also used as nutritious mixture in *in vivo* ecotoxicity tests, meaning that many animals in ecotoxicity studies are fed or supplemented with *Saccharomyces cerevisiae*.

9. Annexes

1	Stability of the inserted genetic material
2	Taxonomy of the donor
3	Confirmation of growth on xylose
4	Antibiotic resistance
5	(b)(4)
6	Inactivation study
7	Laboratory method for inactivation study
8	CoA for *Saccharomyces cerevisiae*
9	Modified *Saccharomyces cerevisiae* nutritional analysis
10	Excerpt of Pilot Plant Report
11	Ethanol Fermentation Raw Material Specifications
12	DDG Amino Acid Analysis Statistics
13	DDG Digestability Statistics
14	Pariza & Cook Decision Tree
15	Literature search
16	Allergen Search
17	MSDS Advanced *Saccharomyces cerevisiae*
18	Yeast Fermentation Raw Material Specifications
19	Stability of yeast fermentation performance

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Section 172: FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION

§ 172.325 - Bakers yeast protein.

§ 172.590 - Yeast-malt sprout extract.

§ 172.896 - Dried yeasts.

§ 172.898 - Bakers yeast glycan.

Section 184: DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

§ 184.1983 - Bakers yeast extract.

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12. Expert Panel Consensus Statement Concerning the Determination of the Safety and Generally Recognized as Safe ("GRAS") Status of the Proposed Use of a Genetically Modified Pentose Fermenting *Saccharomyces Cerevisiae* as a Component of Animal Feed

November 26, 2013

INTRODUCTION

At the request of DSM Nutritional Products, (DSM), a panel (the "Expert Panel") of independent scientists, qualified by scientific training and relevant national and international experience to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information on technical information that supports the Generally Recognized as Safe (GRAS) status of an Inactivated Modified *Saccharomyces cerevisiae* as a nutritional product for use in poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets, based on scientific procedures. The Inactivated Modified *Saccharomyces cerevisiae* will be present in dry distillers' grains with or without solubles at up to 20% of the dry solids. For purposes of this evaluation, "safe" or "safety" as it relates to GRAS within the terms of the Federal Food, Drug, and Cosmetic Act means that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2012).

The organism that is the subject of this GRAS assessment was modified following NIH guidelines and has been accepted by the EPA as safe for large scale industrial fermentation and use for the production of bioethanol. The Modified *Saccharomyces cerevisiae* is derived from the same genus and species as baker's yeast. The nutritional composition of Modified *Saccharomyces cerevisiae* is therefore expected to be very comparable to that of dry baker's yeast already on the market. This was confirmed by comparing the nutritional composition of three lots of Modified *Saccharomyces cerevisiae* with the composition of active dry baker's yeast obtained from the USDA National Nutrient Database (USDA 2013), and commercialized baker's yeast. The Modified *Saccharomyces cerevisiae* is composed of proteins, lipids, carbohydrates, cellulose and minerals. The commercial product for bioethanol production will be sold as either a dry powder produced from a concentrated liquid or the concentrated liquid itself (commercially referred to as 'cream yeast') by DSM Bio-based Products & Services. The inactivated yeast will be a byproduct following distillation of ethanol and a component of Dried Distillers' Grains with or without Solubles (DDG/S) or sold alone as dry yeast.

The modifications to the organism only increased the number of native genes already present and added (b)(4), that is currently approved for use in food production for both animals and humans. The ability to produce novel amino acids

or proteins was not introduced. The gene coding for the non-native enzyme was synthetic and therefore devoid of any extraneous DNA from the template organism.

It is anticipated that the exposure of target animals to the Genetically Engineered, Inactivated Modified *Saccharomyces cerevisiae* will not be higher than the current exposure to classical *Saccharomyces cerevisiae*.

The Expert Panel consisted of the below-signed qualified scientific experts: Mark E. Cook, Ph.D., (University of Wisconsin - Madison), John A. Thomas, Ph.D, F.A.T.S (Indiana University School of Medicine) and Stanley M. Tarka, Jr. Ph.D. (Chair) (The Tarka Group, Inc. and The Pennsylvania State University). Copies of *curricula vitae* evidencing the qualifications of the Expert Panel members are attached.

The Expert Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other sources through November 10, 2013 by DSM Bio-based Products & Services and DSM Nutritional Products and other information deemed appropriate or necessary. The Expert Panel evaluated information on characterization of the source yeast and the production strain of *Saccharomyces cerevisiae* under consideration, manufacturing/production methods, compositional data, specifications and batch analyses, intended use-levels, consumption estimates for the intended uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of this inactivated Modified *Saccharomyces cerevisiae* as compared to classical *Saccharomyces cerevisiae*.

The Expert Panel convened via teleconference and unanimously concluded that for the intended uses of Inactivated Modified *Saccharomyces cerevisiae* produced from a genetically engineered *Saccharomyces cerevisiae*, meeting appropriate food-grade specifications as described in the supporting dossier and manufactured consistent with current Good Manufacturing Practice (cGMP), is GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

SUMMARY OF THE BASIS FOR GRAS STATUS OF THE INTENDED USES OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED

DSM Nutritional Products provided technical information that supports the Generally Recognized as Safe (GRAS) status of Inactivated Modified *Saccharomyces cerevisiae* (also known and commercially marketed as Inactivated "Advanced *Saccharomyces cerevisiae*"), a nutritional product for use in poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats and pets, based on scientific procedures.

DSM Bio-based Products and Services (DSM-BPS) developed this modified yeast; their sister company, DSM Nutritional Products will manufacture and package the product. DSM-BPS will market the product. Modified *Saccharomyces cerevisiae* is used by bio-ethanol facilities for the fermentation of sugars released from plant-based feed stocks, such as corn, into ethanol in a manner similar to the production of beer or grain neutral spirits used for in food and pharmaceutical processing. Inactivated Modified *Saccharomyces cerevisiae* is a heat killed co-product of bio-ethanol production.

Although the yeast was developed for use in bio-ethanol production, DSM-BPS is aware that potential customers may separate the inactivated yeast from the spent stillage for sale as an ingredient for animal feed or it will be dried with the cellulosic material in the stillage and become part of the Wet or Dried Distillers Grains with or without solubles (DDG/S and DDG) depending upon the business practices of the potential customer. These practices are commonly followed today with conventional *Saccharomyces cerevisiae* and the use of the yeast and DDG/S are permitted under feed ingredient definitions of the American Association of Feed Control Officials (AAFCO) as overseen by FDA's Center for Veterinary Medicine, at levels not exceeding good manufacturing procedures (AAFCO O.P. Sections 96 and 27 respectively). DSM-BPS does not intend to exceed the current limits for use of yeast and merely seeks to expand its permitted use to include this genetically engineered strain in an inactivated format.

The subject yeast will be produced in two product forms: a concentrated 'cream yeast' and a dry form commonly referred to as 'active dry yeast'. It is anticipated that the dry form will be the predominant marketed form for bio-ethanol production. Initially the Modified *Saccharomyces cerevisiae* is produced by (b)(4) in a facility that follows food cGMP (21 CFR§ 110). The yeast is then used in bio-ethanol production where it multiplies several times during the fermentation cycle. The yeast is them inactivated during the distillation process. Since the host organism is safe, the incorporated DNA does not encode any known harmful or toxic substances, and the active yeast is inactivated during the distillation process, the resulting inactivated genetically engineered organism is considered to be safe.

Saccharomyces cerevisiae is a ubiquitous microorganism found all over the globe and has been utilized by humans serendipitously and intentionally for the production of bread, beer and wine for over six thousand years. The National Institutes of Health in its Guidelines for Research Involving Recombinant DNA Molecules (Department of Health and Human Services 1986) considers *Saccharomyces cerevisiae* a safe organism.

The safety of the Inactivated Modified *Saccharomyces cerevisiae* has been established based on the published literature and history of use. A comprehensive search of the scientific literature for safety and toxicity information on *Saccharomyces cerevisiae* was conducted by DSM Bio-based Products and Services. The databases searched included Medline, BIOSIS and EMBASE databases as provided by the host STN.

The basis for the GRAS determination of the intended uses of Inactivated Modified *Saccharomyces cerevisiae* produced by (b)(4) in a facility that follows food cGMP from an original *Saccharomyces cerevisiae* unmodified parent strain, (b)(4) (classical wild-type isolate) as a nutrient is through scientific procedures. Additionally, (1) pathogenicity of the taxonomic designation is rare and a outcome of other medical complications, (2) all the modifications are under the genetic control of a *Saccharomyces cerevisiae* promoter and terminator and are integrated in the genome of the yeast, (3) all additional gene sequences have been constructed *in-vitro*, based on the information on the genes as available in gene and/or protein databases, (4) the risk that DNA from the donor organism coding for antibiotic resistance was transferred to the recipient host strain is non-existent, (5) bacterial vectors used in the construction and the selection marker genes have been removed from the final strain (6) it is anticipated that the exposure of target animals to the Genetically Engineered, Inactivated Modified *Saccharomyces cerevisiae* is nonexistent since the organism is killed during the distillation process (7) extensive history of safe use and consumption by animals and humans of inactivated *Saccharomyces cerevisiae* and (8) safety is recognized by multiple regulatory authorities of the parent lineage (FDA, EPA, EFSA). The primary products produced by fermentations utilizing the engineered yeast are yeast biomass and ethanol and in that respect the engineered yeast is not different from the conventional yeast normally used for alcoholic fermentations when inactivated. The Inactivated Modified *Saccharomyces cerevisiae* will be sold either as a single feed ingredient or become part of Distillers Dried Grains with or without Solubles and is intended to be used as a nutrient source. It is substantially equivalent to the host strain, common baker's yeast, from which it is derived, and is therefore Generally Recognized As Safe for use as an ingredient in animal food.

The compositional equivalence of Inactivated Modified *Saccharomyces cerevisiae* is shown by the nutritional analysis of two pilot plant batches and a sub-sample of one commercial batch of "Advanced *Saccharomyces cerevisiae*" as compared to the published nutritional data of active dry baker's yeast and to the nutritional analysis of a sample of commercial baker's yeast. As anticipated, because the "Advanced *Saccharomyces cerevisiae*" is the same in basic molecular composition as conventional yeast, there was no significant difference between the different yeast products.

All relevant information was reviewed, summarized and incorporated into a GRAS dossier, 'THE SAFETY AND THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED' and submitted to the GRAS Panel. The GRAS Panel received information pertaining to the method of manufacture, product specification, analytical data, intended use levels in animal food for avian, ruminant and porcine species, resulting consumption estimates from the intended uses, and any other relevant

data on safety and tolerance-related information. The members of the Expert Panel were Mark Cook, PhD, John A. Thomas, PhD, Fellow, ATS, and Stanley M. Tarka, Jr., PhD (Panel Chair).

SAFETY OF MODIFICATIONS INTRODUCED INTO THE ORGANISM

The original *Saccharomyces cerevisiae* parental strain is also known as baker's yeast or brewer's yeast. The unmodified (b)(4) was identified in the laboratory by (b)(4) and was deposited at (b)(4) lists the unmodified source organism as *Saccharomyces cerevisiae* (b)(4) . Subsequently, (b)(4) was obtained by and modified at (b)(4) . The resulting modified strain was designated as (b)(4) .

The nature of the unmodified parent strain, (b)(4) , is that it is a congenic member of the (b)(4) family as described by (b)(4) . The parent strain, (b)(4) was a classical wild-type isolate.

There are no known pathogens associated with this strain of *Saccharomyces cerevisiae* , thus, the designation is sufficient to distinguish the organism from species that exhibit pathogenicity under the genus *Candida*.

- *Saccharomyces cerevisiae* has been modified to (b)(4) (b)(4)

All the modifications are under the genetic control of a *Saccharomyces cerevisiae* promoter and terminator and are integrated in the genome of the yeast.

All additional gene sequences have been constructed *in-vitro*, based on the information on the genes as available in gene and/or protein databases. The risk that DNA from the donor organism coding for antibiotic resistance was transferred to the recipient host strain is non-existent.

Bacterial vectors used in the construction and the selection marker genes have been removed from the final strain.

The (b)(4) genes enable the yeast to metabolize C5 sugars in addition to the normal C6 sugars, and thus produce ethanol from grain and grain by-product feedstocks.

Nevertheless, the primary products produced by fermentations utilizing the engineered yeast are yeast biomass and ethanol and in that respect our engineered yeast is not different from the conventional yeast normally used for alcoholic fermentations.

PRODUCTION STRAIN

The strain phenotype is understood to refer to the expression of the genes of the organisms as well as the influence of environmental factors and random variation.

The unique characteristic that differentiates (b)(4) is its ability to efficiently ferment xylose in plant hydrolysates containing hemicellulose derived pentoses and acetic acid. To obtain this, the strain has an (b)(4) That is what distinguishes the strain from other strains.

The strain phenotype is (b)(4)

Regarding phenotypic changes resulting from modifications to the microorganism, all gene sequences, except for (b)(4), were sourced from the host strain *Saccharomyces cerevisiae*. As previously described, (b)(4) into ethanol.

MANUFACTURING

Modified *Saccharomyces cerevisiae* is manufactured by DSM Nutritional Products following Good Large Scale Fermentation Practices and is in compliance with the applicable sections of 40 CFR §725. Inactivated Modified *Saccharomyces cerevisiae* is a co-product of the manufacture of the bio-fuel industry. Inactivated Modified *Saccharomyces cerevisiae* will enter the animal food chain as a by-product of ethanol production from grains, such as corn, wheat, barley, rye and other forage materials. It is anticipated that the substance will be isolated from the stillage of the Bio-fuel plant and sold a nutritive ingredient or it will be a component of the dried stillage commonly referred to as 'dried distillers' grains' (DDG). The yeast and the enzymes it expressed are inactive in the feed ingredient.

The Modified *Saccharomyces cerevisiae* was developed for the purpose of providing a robust yeast capable of metabolizing C5 and C6 sugars that could be easily added to the fermentation tanks of fuel ethanol producers. To accomplish this goal, the modified organism is produced in large quantities and packaged in suitable containers in a manner similar to standard baker's yeast as illustrated in the following diagrams. The organism is grown in a (b)(4) process; (b)(4).

(b)(4)

(b)(4)

The Modified *Saccharomyces cerevisiae* is designed for the optimal production of bio-ethanol. The inactivated yeast is recovered from the stillage of the bio-fuel plant along with the unfermented plant matter in the same manner as traditional fermentation processes.

Under the current processing methods followed in a fuel ethanol facility, the Modified *Saccharomyces cerevisiae* will be exposed to lethal conditions in the distillation process step ensuring that only non-viable cells are incorporated into the feed of animals. The temperature and exposure time in the distillation step are above the generally accepted maximum for enzyme stability. The added enzymes were not selected for temperature stability and to the knowledge of DSM are not thermo-stabile. Data provided in the GRAS dossier contains the results of experiments that confirm that the organism is destroyed when exposed to normal bio-fuel distillation conditions. The references 'Alcohol Textook, 'chapters 16 and 19 (Richards 2009, Earnest, Snyder et al. 2009) provided additional information regarding fermentation plant operations that support the premise that the yeast will be killed during downstream processing at the biofuel plant.

COMPOSITION

The Modified *Saccharomyces cerevisiae* is composed of proteins, lipids, carbohydrates, cellulose and minerals. The commercial product for bioethanol production will be sold as either a dry powder produced from a concentrated liquid or the concentrated liquid itself (commercially referred to as 'cream yeast') by DSM Bio-based Products & Services. The inactivated yeast will be a component of Dried Distillers' Grains with or without Solubles (DDG/S) or sold alone as dry yeast.

Certificates of analysis for typical Modified *Saccharomyces cerevisiae* batches demonstrate consistency of production lots to established specifications (Table 1).

Table 1 Modified *Saccharomyces cerevisiae* liquid concentrate specifications and batch to batch variation

Parameter	Units	Specification (cream yeast)	Pilot Batch Rpt 1202 CRY	Batch C177000	Batch C177001
Appearance		Turbid cream yeast (concentrated broth) with a white/beige color	complies	complies	complies
Dry matter	%	(b)(4)			
pH					
Glucose	g/l				
Microbial contamination: total bacterial count	CFU/mL				
Microscopic observation in the EOF broth		conform	complies	complies	complies

The Modified *Saccharomyces cerevisiae* is derived from the same genus and species as baker's yeast. The nutritional composition of Modified *Saccharomyces cerevisiae* is therefore expected to be very comparable to that of dry baker's yeast already on the market. This was confirmed by comparing the nutritional composition of three lots of Modified *Saccharomyces cerevisiae* with the composition of active dry baker's yeast obtained from the USDA National Nutrient Database (USDA 2013), and the commercialized baker's yeast. Any difference in fiber content is due to methods of expressing fiber. In the active dry baker's yeast reported on the USDA database, the total dietary fiber content was determined, whereas only crude fiber content was measured in Modified *Saccharomyces cerevisiae* and a commercial baker's yeast (Table 2).

Table 2 Nutritional properties of the Modified *Saccharomyces cerevisiae*

Parameter	Units	Leavening agent, active dry baker's yeast*	Lot 1	Lot 2	Lot3	Baker's yeast (b)(4)
			1315 A	K160 A	1313 EF	
Dry Matter	%	94.9	92.2	92.1	89.7	95.9
Energy	kcal/Kg	3250	3218	3253	3145	3405
Protein	%	40.44	46.10	46.15	46.01	44.09
Fat	%	7.61	6.16	6.09	5.77	4.17
Crude Fiber	%	26.9**	<0.2	<0.2	<0.2	<0.2
Ash	%	NA	9.06	7.85	8.10	4.57
Ca	%	0.03	0.005	<0.004	0.004	0.09
P	%	0.637	1.88	1.54	1.33	0.79
K	%	0.955	2.26	2.54	2.16	1.55
Mg	%	0.054	0.27	0.18	0.19	0.09
Zn	ppm	79	121	106	93	80
Mn	ppm	NA	8	7	11	5
Cu	ppm	NA	7	9	12	2
Fe	ppm	22	49	11	68	54

NA: not available

* USDA National Nutrient Database for Standard Reference Release 26

** Total dietary fiber

Ash content is driven by the media composition. Baker's yeast has been commercially produced for over 75 years and the media composition has been optimized to be the least expensive formulation that allows for maximum cell mass in the shortest period of time. The media formulation for the Modified *Saccharomyces cerevisiae* has not been optimized at this point in time and may be too rich in minerals. DSM anticipates that the concentration of the media components may be altered in the future.

The difference in protein is driven by differences in the parent strain, media composition and the differences in the fermentation process. The Modified *Saccharomyces cerevisiae* has been developed to efficiently perform in biofuel substrates such as crude corn digests. Baker's and brewer's yeast used in feed are valued for their protein content and an increase in protein would be welcomed by the feed industry.

High protein levels (up to 60% on a dry matter (dm) basis is possible) lead to high yeast activity in bread applications, however they lead to a lower shelf life of the (fresh) yeast. In the case of dry yeast, an additional factor is that high protein yeast will not sufficiently survive the drying process. The yeast protein in baker's yeast products may vary from 40% to 60-65% on dm. For active dry yeast it may vary from 40-55% on dry matter (Reed, Peppler 1973).

The specification in the 2013 AAFCO OP definition 96.1 for dried yeast (Association of American Feed Control Officials 2013) is that the organism is *Saccharomyces cerevisiae* and has a minimum protein content of 40%. The Modified *Saccharomyces cerevisiae* that is the subject of this GRAS assessment easily meets this requirement.

Three batches of Dried Distillers' Grains with Solubles (DDG/S) containing the Inactivated Modified *Saccharomyces cerevisiae* were produced via fermentation of corn in a commercial bio-fuel processor following their normal process. The use of the Modified *Saccharomyces cerevisiae* in a bioethanol fermentation did not alter the specifications of the resulting dried distillers' grains with solubles (DDG/S). Although the dry yeast itself currently has an elevated protein and ash content compared to traditional baker's yeast, those differences do not alter the composition of the commercial feed ingredient in a significant manner (Table 3).

Table 3 Physical properties of the Dried Distillers' Grains with Solubles containing the Modified *Saccharomyces cerevisiae*

Parameter	Units	Specification	Lot 1 27-510	Lot 2 27-506	Lot3 27-502
Physical state		granules	granules	granules	granules
Solubility		complete and clear	complete and clear	complete and clear	complete and clear
Protein	%	30-35	32.69	33.68	31.69
Loss on drying	%	max 6	5.12	4.89	3.81
Residue on ignition	%	max 5	3.92	3.72	3.22
carbohydrates	%	max 50	44.5	46.1	44.4

These data collectively support the use of Inactivated Modified *Saccharomyces cerevisiae* as a suitable nutritional ingredient for animal feed.

Stability

The Modified *Saccharomyces cerevisiae* has been developed for use in the fermentation of biomass-derived carbohydrates for the purpose of producing fuel ethanol. The organism is not designed for nor is it expected to be stored for long periods of time before use in the biofuel plant. However, a stability study has demonstrated that the Inactivated Modified *Saccharomyces cerevisiae* retains its fermentative capacity after 6 months at 4° C, the recommended storage temperature.

INTENDED USE, USE LEVEL AND EXPOSURE

It is not expected that the amount of Inactivated Modified *Saccharomyces cerevisiae* in DDG/S or feed will be higher than the amount of classical *Saccharomyces cerevisiae* already found in feed or DDG/S, as a byproduct of conventional ethanol distillation. It was estimated that dried yeast represent 20% by weight of the dried distillers' solubles (Liu 2011).

The expected animal intake of DDG/S containing Inactivated Modified *Saccharomyces cerevisiae* is equal or less than the current intake of DDG/S containing classical *Saccharomyces cerevisiae* derived from starch-based biomass. These intakes depend on the nutritional needs of the specific target animals and are summarized in the table below (Table 4).

Since the nutritional content of Inactivated Modified *Saccharomyces cerevisiae* is very similar to classical *Saccharomyces cerevisiae* (see Table 2), the intake levels of Inactivated Modified *Saccharomyces cerevisiae* by the target animals will not exceed what is expected from classical *Saccharomyces cerevisiae*.

The following table (Table 4) illustrates the maximum percentage of modified yeast in the diet based upon the literature for DDG/S in the diet and the quantity of yeast in DDG/S (Liu 2011).

Table 4 Intake levels of DDG/S in feed of target animals

Species / Class	DDG/S Maximum % of Diet	Modified *Saccharomyces cerevisiae* Maximum % of Diet
Swine (Shurson, Spiehs et al.)		
Nursery pigs (>15 lbs)	25	5
Grow-finish pigs	20	4
Developing gilts	20	4
Gestating sows	50	10
Lactating sows	20	4
Boars	50	10
Poultry (National Corn Growers Association 2008)		
Chicken - Broilers	10	2
Chicken – Layers	15	3
Turkeys (grow/finish)	15	3
Bovines (Weiss, Eastridge et al.)		
Beef cattle	25	5
Dairy cows	25	5

It is anticipated that the Inactivated Modified *Saccharomyces cerevisiae* will be utilized in animal feed either as an individual feed ingredient similar to traditional dried yeast or as a component of Distillers' Dried Grains with or without Solubles (DDG/S). As an individual feed ingredient the material will be added to the feed of poultry, swine, bovines, minor species and pets at levels not to exceed good manufacturing practices.

The table below (Table 5) presents the possible range of use and exposure of Modified *Saccharomyces cerevisiae.* It will be used alone or as a component of an ingredient (Dried Distillers' Grains) in the feed of the following production animals as part of a complete ration.

Table 5 Proposed use level and exposure of Production Animals to Modified *Saccharomyces cerevisiae*

Species /Class	Maximum Feed Consumption* (Kgs per day)	Maximum Level of Modified yeast in Feed from Table 4	Maximum intake of Modified yeast (Kg per day)	Number of days of consumption	Total exposure Kgs of yeast	Exposure (mg Modified yeast/kg BW/day+)
Broiler Chickens	0.11	2%	0.0022	48	0.106	1.22
Laying Hens	0.1	3%	0.003	365	1.09	1.67
Breeding Hens	0.1	3%	0.003	365	1.09	1.67
Piglet	0.2	5%	0.01	56	0.56	1.10
Sows	1.8	10%	0.18	365++	65.7	1.20
Lactating Sows	5.17	10%	0.517	579++	299.3	3.45
Finisher	4	4%	0.16	126	20.2	1.31
Turkeys	0.2	3%	0.006	140	0.84	0.63
Beef Cattle	10.6	5%	0.53	180	95.4	0.97
Dairy Cows	22	5%	1.1	1460	1,606	2.09

* (Subcommittee on Feed Intake, Committee on Animal Nutrition, National Research Council 1987)

+ Average weight at slaughter

++ (Stalder, Engblom et al. 2009)

SAFETY ASSESSMENT OF THE PROPOSED USE OF A GENETICALLY ENGINEERED PENTOSE FERMENTING *SACCHAROMYCES CEREVISIAE* AS A COMPONENT OF ANIMAL FEED

Antibiotic Resistance

The final production strain does not contain any Antibiotic Resistance genes. The strain is susceptible to antibiotics and to anti-fungals. When tested, the genetic changes introduced into the Inactivated Modified *Saccharomyces cerevisiae* do not affect antifungal susceptibility.

A literature search did not produce any articles related to resistance to antibiotics. Since the inserted genetic elements in this case do not appear to possess any intrinsic hazard potential, data were provided for the species in general based on the rationale that the gene modification to the organism was not shown through a literature search to produce an effect or yield different results from the parental strain. For this reason, Expert Panel concurs with DSM that it is appropriate to use the parental strain, *Saccharomyces cerevisiae,* as a surrogate strain for gathering information and assessing the effect of the modified strain on antibiotic resistance and tolerance to metals and pesticides.

Based on the absence of demonstrated adverse effects for the parental strain and for the inserted intergeneric sequence, it is reasonable to conclude that the modified strain is not expected to be any different from other well-known *Saccharomyces cerevisiae* strains commonly found in nature.

Action as a Potential Vector for Pathogens

A search of the literature did not identify any articles demonstrating (b)(4) p ermits the production strain to act as a vector of pathogens. A null result was not unexpected since (b)(4) has been used in industry and food processing for years (b)(4) without any adverse effect. There are no studies that the submitter could locate that would indicate the donor strain itself, (b)(4) acts as a vector for pathogens. Because the gene modification to the organism was not shown through a literature search to yield different results from the parental strain, surrogate information on the recipient strain is offered for the purpose of evaluating the anticipated behavior of the production strain. Based on the information provided in EPA's February 1997 Final Risk Assessment (the Environmental Protection Agency 1997), the production strain is not expected to be a vector for pathogens. The production strain is not expected to act as a vector of any pathogen such as *Escherichia coli* or *Clostridium botulinum.*

Safety Assessment of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has an extensive history of use in food processing. Also known as Baker's Yeast or Brewer's Yeast, this organism has been used from as early as the Stone Age (about 9000 BC) as leavening for bread and as a fermenter of alcoholic beverages (Tucker, Woods 1995). But it was only between 1857 and 1863 that Louis Pasteur demonstrated the role played by yeasts, as the micro-organism responsible for fermentation. He noted at that time that one type of living cells was responsible for the fermentation of bread, beer, wine, cider corresponding to a population of a microscopic fungus, *Saccharomyces cerevisiae*. He discovered that a number of varieties of *Saccharomyces cerevisiae* exist in nature and are more or less adapted to the different fermentations (Pretorius 2000). *Saccharomyces cerevisiae* is considered Generally Recognized as Safe through its use in the brewing, baking and winemaking

industry and is the subject of several GRAS Notices, 120, 175, 239, 260, 284, 350, 353 (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175, CFSAN / Office of Food Additive Safety 2008, GRN 000260, CFSAN / Office of Food Additive Safety 2008, GRN 000239, CFSAN / Office of Food Additive Safety 2009, GRN 000284, CFSAN / Office of Food Additive Safety 2010, GRN 000350, CFSAN / Office of Food Additive Safety 2010, GRN 000353).

In food production today the use of yeasts is not limited to the production of bread or fermented beverages. Since the 1930s yeast extract products were developed to enhance the flavor of a variety of products such as soups, sauces and ready-to-eat meals. Moreover, because of their nutritional characteristics (protein, vitamin, mineral and amino acid content), preparations of living cells or yeast cell walls have been commercialized as food supplements. Yeast is recommended for dietary supplementation for patients with diabetes type 2, diarrhea, high cholesterol and fatigue due to its high chromium and vitamin B content (see www.vitacost.com). In addition, the organism is widely used for the production of macromolecular cellular components such as lipids, proteins, enzymes, and vitamins (see www.vitacost.com; Moyad 2007, Moyad 2008).

Saccharomyces cerevisiae is a ubiquitous yeast. It is found on our bodies and in the air that we breathe, and it is naturally present on and in foods that man and animals eat regularly.

In its safety evaluation of carbohydrase from *Saccharomyces cerevisiae*, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered that *Saccharomyces cerevisiae* belongs to the group of micro-organisms traditionally accepted as constituents of foods or normally used in the preparation of foods (Joint FAO/WHO Expert Committee on Food Additives 1971).

In 1997, the US Environmental Protection Agency (EPA) issued their safety assessment of *Saccharomyces cerevisiae* and concluded that "*S. cerevisiae* is an organism which has an extensive history of safe use. Despite considerable use of the organism in research and the presence of *S. cerevisiae* in food, there are limited reports in the literature of its pathogenicity to humans or animals, and only in those cases where the human had a debilitating condition. Tests for the factors associated with the virulence of yeasts (i.e., phospholipases) indicate that this organism is nonpathogenic. The organism has not been shown to produce toxins to humans." (Environmental Protection Agency, 1997) *Saccharomyces cerevisiae* was therefore included as a recipient microorganism at § 725.420 for the tiered exemption.

According to scientific experts, a nontoxigenic organism is defined as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism is defined as "one that is very unlikely to produce disease under ordinary circumstances." (Pariza, Foster 1983).

The European Food Safety Agency (EFSA) concluded that baker's yeast (*Saccharomyces cerevisiae*) fits this definition of nontoxigenicity and nonpathogenicity and that it is also not a major food allergen. EFSA therefore gave baker's yeast a Qualified Presumption of Safety (QPS) status (Opinion of the Scientific Committee, 2007). This means that in Europe, baker's yeast can be safely used in food and feed production and is exempt from the need for further safety assessment.

The FDA considers *Saccharomyces cerevisiae* and several derived products safe for consumption. Indeed, FDA has approved dried yeast as an ingredient for food (21 C.F.R. §172.896), and baker's yeast extract has been affirmed by the FDA as a GRAS flavoring agent and adjuvant (21 C.F.R. §184.1983). The FDA has also approved various yeast-derived products for their use in food. These include Baker's yeast protein (21 C.F.R. §172.325), Yeast-malt sprout extract (21 C.F.R. §172.590) and Baker's yeast glycan (21 C.F.R. §172.898).

In addition, GRAS notifications have been submitted and accepted by FDA with no questions for the use of genetically modified yeasts as starter cultures for wine (CFSAN / Office of Food Additive Safety 2003, GRN 000120, CFSAN / Office of Food Additive Safety 2005, GRN 000175).

Safety Considerations Due to the Nature of Modifications of *Saccharomyces cerevisiae*

The US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420). This exemption is permitted because genetically engineered strains of the species were found to have no adverse effects on man or the environment. They also determined that the introduction of intergeneric material will not increase the potential for adverse effects, provided that the genetic material is limited in size, well characterized, free of certain sequences and poorly mobilizable (Environmental Protection Agency, 1997).

For DSM's Modified *Saccharomyces cerevisiae*:

- the only additional heterologous genetic material was (b)(4) and the coding sequence of the inserted gene is limited in size;
- the additional genetic material is completely characterized as it was made synthetically;
- the additional genetic material by default is free from sequences of concern, and the additional genetic material is poorly metabolizable as it is integrated in the genome of the host and lacks sequences which would allow it to be mobilized;
- no antibiotic resistance genes are present;
- no toxigenic genes were inserted;
- no antibiotic production genes were inserted.

The gene insertion was not random but targeted and the locations are known as illustrated in sections 2.3.5 and 2.4 of this dossier. The entire genome of *Saccharomyces cerevisiae* is known and it is free of toxin producing DNA sequences (see http://www.yeastgenome.org/ for the entire genome). It is not possible to induce the formation of a new attribute utilizing the techniques described in the organism construction sections of the dossier.

The Modified *Saccharomyces cerevisiae* that is the subject of this GRAS Notice is also the subject of the Microbial Commercial Activity Notice J13-0007 which was dropped from their review in June 2013. DSM also assessed the safety of the Inactivated Modified Yeast as it relates to its use in foods according to the decision tree guidelines developed by Pariza and Johnson (2001) for food enzymes and later modified by Pariza and Cook (2010) for animal feed enzymes derived from GE organisms. The test article was shown to be acceptable for feed use. In addition, the

entire organism is boiled in acidic aqueous fermentation broth during the distillation step in biofuel production and destroyed.

The Expert Panel critically evaluated published data and a third party assessment by the European Food Safety Agency (Opinion of the Scientific Committee, 2007 on the safety of *Saccharomyces cerevisiae*). Particular attention in the EFSA focused on reports of the role of *S. cerevisiae* as an aetiological agent of invasive infection in "fragile" populations. EFSA concluded "*The body of knowledge is considered as sufficient (long history of safe use) with only 92 cases of pathogenic cases involving S. cerevisiae reported in total (15 cases diagnosed before 1990); all patients had at least one condition facilitating the opportunistic development of S. cerevisiae. S. bayanus and S. pastorianus are used in wine and beer production. There are no foodborne infection issues for these species.* On the basis of their assessment, EFSA classified *Saccharomyces cerevisiae* as a QPS (Qualified Presumption as Safe) organism, a non-pathogen and not a major food allergen. *Saccharomyces* has been an approved component of feed since at least 1951 and there are no literature reports that identify *Saccharomyces cerevisiae* as toxic for animals. As stated earlier, this means that it can be safely used in food and feed without additional safety testing.

DSM also performed a comprehensive literature search and confirmed that although the cases of human infection with *Saccharomyces cerevisiae* are rare, some isolates have pathogenic potential. Some clinical isolates are notably known to be virulent in humans. In addition, several reports evaluating the safety of commercial *Saccharomyces cerevisiae* strains have clearly identified the subtype *boulardii* as a potential pathogen, in particular towards immunocompromised patients (Graf and Gavazzi, 2007).

Although very few scientific reports are available on potential adverse effects of *Saccharomyces cerevisiae* for animals, the few toxicity studies that were performed on rats, mice or monkeys with the yeast or with the yeast biomass do not suggest that these animals display a stronger sensitivity towards *Saccharomyces cerevisiae* than humans.

Recent efforts have focused on attempting to characterize the virulence traits related to *Saccharomyces cerevisiae, thus* enabling the means to better identify the potential pathogenic strains. The phenotypic traits of virulence of pathogenic strains are believed to be due to their ability to grow at high temperature (38-42◦C), their adherence and their ability to invade host cells, and their ability to produce and secrete degradative enzymes (i.e. proteinase and phospholipase). This search did not identify any reports of isolates of *Saccharomyces cerevisiae* able to produce toxins against animals or humans, although several studies have shown the ability of the yeast to produce toxins against other yeasts (the so-called killer toxins).

The Expert Panel concluded that DSM's literature search does not alter the conclusions previously made by several bodies of experts that *Saccharomyces cerevisiae* can be considered as a safe microorganism and a non-pathogen, although some of the yeast isolates should be regarded as opportunistic pathogens of low virulence.

Presence of Methyl Glyoxal

In metabolically active yeast cells, methylglyoxal may be formed during normal cell metabolism. Methylglyoxal synthase catalyzes the reaction from dihydroxyacetonephosphate to methylglyoxal. Hashimoto et. al (1997) found that genetically engineered *Saccharmoycese cerevisiae* strain DKD-5D-H produced an increased amount of methylglyoxal, a toxic 2-oxoaldehyde, compared to

the wild-type yeast cells. This increase was reportedly due to the genetic manipulations involving the enzymes phosphoglucose isomerase, phosphofructokinase and triosephosphate isomerase.

In the Inactivated Modified Yeast, overexpression of the *GLO1*-gene is brought about. To this end, the *Saccharomyces cerevisiae GLO1*-gene is expressed under control of the PGK1-promoter. The *GLO1*-gene encodes monomeric glyoxalase I, which catalyzes the detoxification of methylglyoxal (a by-product of glycolysis) via condensation with glutathione to produce S-D-lactoylglutathione. The Expert Panel noted that DSM explains in the GRAS dossier that overexpression of the GLO1-gene helps in lowering the intracellular glyoxal levels and therefore, this is not a concern.

Human Exposure

Human exposure is anticipated to be limited to individuals handling the material during packaging at the yeast production facility, the bio-ethanol facility, in the manufacture of feed, on the farm, and from consumption of the target animals. The use of personal protective equipment and compliance with normal occupational safety practices will minimize exposure.

No toxicokinetic studies on *Saccharomyces cerevisiae* were identified by DSM. However, *Saccharomyces cerevisiae* will be metabolized during animal digestion into essential compounds consisting mainly of proteins and carbohydrates. Amino acids and peptides are important building blocks in the human body. Carbohydrates are indispensable for storage or transport of energy, and they also play a role in immune function, blood clotting and development.

The Expert Panel concurred and concluded that *Saccharomyces cerevisiae* is in general considered to be of very low toxicity for humans. This conclusion is based on human evidence coming from a long history of safe use, on its composition, and on the fact that it is readily biodegradable in the human gastro-intestinal tract. In the public domain, toxicity studies on *Saccharomyces cerevisiae* are limited. However, due to low risk expected for man and the environment, the US EPA has included *Saccharomyces cerevisiae* as a recipient microorganism is exempt from TSCA Section 5 review and suitable for a Tier 1 exemption (40 CFR §725.420).

Allergenicity Concerns

Inhalation allergy towards *Saccharomyces cerevisiae* has been described by Baldo and Baker (Baldo, Baker 1988). This type of allergy is predominantly observed in bakers.

Regarding oral allergy against *Saccharomyces cerevisiae,* only one possible case is known (Pajno, Passalacqua et al. 2005). An atopic 6-year old boy experienced generalized urticaria and asthma after eating pizza and bread fresh from the oven (within one hour. In its assessment of *Saccharomyces cerevisiae* for a classification as QPS (Qualified Presumption as Safe) organism, the European Food Safety Agency (EFSA) concluded that *Saccharomyces cerevisiae* is not a major food allergen (Opinion of the Scientific Committee, 2007).

In addition, DSM's also reported on their assessment of the impact of the modifications made on the *Saccharomyces cerevisiae* portion of DDGS was evaluated by applying the guidelines on testing the potential allergenicity of genetically modified foods as presented by the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (Food and Agriculture Organization of the United Nations/World Health Organization 2001, Food and Agriculture Organization of the United Nations/World Health Organization 2009).

The Inactivated Modified *Saccharomyces cerevisiae* has been engineered to (b)(4) but no foreign DNA sequences known to code for allergenic proteins were inserted into the organism.

Regarding the above, it is concluded that the inserted gene protein product has no relevant match with known (food) allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

Environmental **Impact**

The Expert Panel also noted and agreed that *Saccharomyces cerevisae* is exempted from TSCA Section 5 PreMarket Notification by the Environmental Protection Agency (40 CFR §725.420, the Environmental Protection Agency 1997). The EPA has reviewed and approved the use of this genetically engineered *Saccharomyces cerevisiae* for biofuel production under Microbial Commercial Activity Notice (MCAN) J11-0001. Another genetically engineered yeast, *Pichia pastoris*, has also been approved under MCAN J04-0003. DSM has filed a Tier 1 exemption notice with the EPA which was accepted by the agency in December 2011 and a Microbial Commercial Activity Notice which was accepted on 18 March 2013 and dropped from review on 12 June 2013.

Inactivated Modified *Saccharomyces cerevisiae* is a GRAS substance and per 21 CFR §25.32, foods, food additives and color additives, including GRAS substances, are categorically excluded from the requirement to provide an environmental impact statement or an environmental assessment.

Inactivated Modified *Saccharomyces cerevisiae* will be added directly to animal food and is intended to remain in the food through ingestion by animals and it is not intended to replace macronutrients in animal food.

There is no information on the ecotoxicity of Modified *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* in the public domain. However, because of the macro-composition of Modified *Saccharomyces cerevisiae*, consisting mainly of proteins and carbohydrates, it can be expected that the substance is readily biodegradable. Therefore, no toxicity for invertebrates, aquatic plants and fish is expected, since the substance will be degraded by bacteria in the water. In addition, as was previously indicated, *Saccharomyces cerevisiae* has been widely fed to poultry, cattle and other livestock due to its nutritious properties and is thus of very low toxicity. It is also used as nutritious mixture in *in vivo* ecotoxicity tests, meaning that many animals in ecotoxicity studies are fed or supplemented with *Saccharomyces cerevisiae*.

CONCLUSION

We, the undersigned independent qualified members of the Expert Panel, have individually and collectively critically evaluated the data and information summarized above, as well as other data and information that we deemed pertinent to the safety of the intended conditions of use of the genetically engineered pentose fermenting *Saccharomyces cerevisiae*, referred to in this document as Inactivated Inactivated Modified *Saccharomyces cerevisiae*, produced during bio-fuel production from plant- based materials and meeting appropriate established specifications is safe for consumption by production animals, minor species and pets. Modified *Saccharomyces cerevisiae* is manufactured following Good Large Scale Fermentation Practices and is in compliance with the applicable sections of 40 CFR §725. Inactivated Modified *Saccharomyces cerevisiae* will enter the animal food chain as a by-product of ethanol production from grains, such as corn, wheat, barley, rye and other forage materials. It is anticipated that the substance will be isolated from the stillage of the bio-fuel plant and sold as a nutritive ingredient or it will be a component of the dried stillage commonly referred to as 'dried distillers' grains' (DDG).

We further conclude that the proposed uses of Inactivated Modified *Saccharomyces cerevisiae* meeting appropriate feed-grade specifications presented in the supporting dossier and produced consistent with Good Large Scale Fermentation Practices, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

Mark E. Cook — Dec 12, 2013

Mark E. Cook, Ph.D.
University of Wisconsin (Panel Member)

Date

Stanley M. Tarka — 13 December 2013

Stanley M. Tarka, Ph.D.
The Tarka Group, Inc. (Chair)

Date

John A. Thomas — Dec. 11, 2013

John A. Thomas, Ph.D., F.A.T.S.
Indiana University School of Medicine (Panel Member)

Date

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Section 172: FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION

§ 172.325 - Bakers yeast protein.

§ 172.590 - Yeast-malt sprout extract.

§ 172.896 - Dried yeasts.

§ 172.898 - Bakers yeast glycan.

184: DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

§ 184.1983 - Bakers yeast extract.

(b)(4)

Trull, Chelsea T-3

From: Wong, Geoffrey K
Sent: Monday, December 12, 2016 2:37 PM
To: Trull, Chelsea
Cc: Betancourt, Angela; Hendricks, Thomas T
Subject: FW: Requested changes to AGRN#20
Attachments: DSM GRAS Notice-Modified_yeast-New page 3.pdf; DSM GRAS Notice-Modified_yeast-New page 4.pdf; DSM GRAS Notice-Modified_yeast-New page 9.pdf; DSM GRAS Notice-Modified_yeast-New page 90-new_sec_6.4.pdf

Chelsea, Please log these pages as an amendment to M000045. Thanks, Geoff

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From: La-Marta, James [mailto:James.LaMarta@dsm.com]
Sent: Monday, December 12, 2016 2:20 PM
To: Wong, Geoffrey K; Betancourt, Angela
Subject: Requested changes to AGRN#20

Dear Mr. Wong,

Attached are the page modifications you requested this morning regarding DSM's GRAS Notice.
I had to change the Table of Contents to account for the new section on the safety of the marketed forms requested by Dr. Betancourt, they are pages 3 &4.
Fortunately, the new section fit into an open part of the page and did not require a whole new page with the 'a' designation.
This revised page is 90.

You also asked for a revision of page 9, which is attached.
This contains the requested revision related to pets in section 1.5 and the revision of section 1.6.

I hope these revisions meet your needs.

Kind Regards,

Jim La Marta, Ph.D.

James La Marta| Senior Manager | Regulatory Affairs | DSM Nutritional Products | 45 Waterview Boulevard | Parsippany , NJ 07054 - 1298| United States of America | T: 1-973-257-8347 | F: 1-973-257-8414 | M: (b) (6) |

1.7 GRAS Exemption Claim

DSM Innovation Inc. provided the appropriate information on the safety and utility of the notified substance to an independent panel of qualified experts, the GRAS Panel for their evaluation. The enclosed dossier contains the information on the identity of the production organism, manufacture of the commercial product and information supporting the safety of its intended use. Also included are copies of the pertinent literature and other supportive data.

DSM Innovation Inc. concluded that Inactivated Modified *Saccharomyces cerevisiae* meeting appropriate animal food-grade specifications as described in this dossier and manufactured consistent with current Good Manufacturing Practices (cGMP), is Generally Recognized As Safe (GRAS) based on scientific procedures and is therefore exempt from the requirement for premarket approval noted in Section 201 (s) of the Federal Food Drug and Cosmetic Act.

1.8 Availability of information for FDA review

The data and information that are the basis for DSM's GRAS determination are available for the FDA's review and copying upon request during normal business hours at:

DSM Innovation, Inc.
BioProducts & Services Division
45 Waterview Blvd
Parsippany, NJ 07054
Tel: 973-257-8500

James La Marta, Ph.D., CFS

Date: 25 March 2016

organism is boiled in acidic aqueous fermentation broth during the distillation step in biofuel production and destroyed as seen in Figure 2-13. The decision tree is in Annex 14.

6.4 Safety of the market forms of inactivated modified yeast

The primary means by which the inactivated modified yeast will be incorporated into animal food is via distillers dried grains with or without solubles that will be a by-product of ethanol fermentation. Alternately, it is possible that an ethanol manufacture may choose to isolate the yeast from the fermentation media components and offer the inactivated yeast itself as a nutritional ingredient for animal food consistent with the currently accepted use of other yeasts, yeast extracts and hydrolysates listed in the AAFCO Official Publication. As noted in section 3.8 of this dossier, there is no meaningful difference between the nutritional composition of the inactivated modified yeast and that of a commercial brand of baker's yeast or the example from the USDA database. Yeast is primarily used in animal food to boost the protein level and the inactivated modified yeast has a slightly high protein level, which would mean its use rate would be lower than that of a commercial baker's yeast to obtain the same effect.

In the case where the inactivated modified yeast is a component of the DDG/S, table 4-2 clearly shows that there is no meaningful difference between DDG/S with conventional yeast and that containing the inactivated modified yeast. Once again, the only difference is a slightly higher protein concentration in the samples containing the inactivated modified yeast.

Therefore, both means of incorporating the inactivated modified yeast into food for pets and production animals: poultry (broilers, layers and breeding chickens; turkeys), swine (piglets, growers, finishers, gestating and lactating sows), bovines (beef and dairy), fish (salmonoids, catfish, tilapia) and minor species such as ducks, quail, sheep, goats does not create a safety concern.

6.5 Exposure for target animals

It is anticipated that the Inactivated Modified *Saccharomyces cerevisiae* will be utilized in animal feed either as an individual feed ingredient similar to traditional dried yeast or as a component of Distillers' Dried Grains with or without Solubles (DDG/S). As an individual feed ingredient the material will be added to the feed of poultry, swine, bovines, minor species and pets at levels not to exceed good manufacturing practices.

The table below presents the possible range of use and exposure of Modified *Saccharomyces cerevisiae*. It will be used alone or as a component of an ingredient (Dried Distillers' Grains) in the feed of the following production animals as part of a complete ration.

Table 6-1 Proposed use level and exposure of Production Animals to Modified *Saccharomyces cerevisiae*