

Chapter 4: Safety Evaluation of Foods and Food Ingredients Derived from Microorganisms

1. INTRODUCTION

Microbes have been an important part of food preparation for millennia. They are consumed directly, and are in fact essential, in familiar foods such as cheese, bread, and yogurt as well as in a variety of Oriental foods such as natto and tempeh. Products of microbial fermentation have a long history of safe use in beer, wine, soy sauce, and vinegar preparation. Desirable microorganisms are also used simply as tools to produce food ingredients. Among these are alcohol, food acids, proteins, enzymes, fat, vitamins, and flavors. In most of these cases, the microorganisms and their products are not present in sufficient quantity to make a substantial contribution to the product's overall nutrient composition, however, consideration has been and still is being given to producing microorganisms for use in food and feed as sources of protein, fat, and vitamins. This application is largely dependent on economics, that is, the cost of the substrate on which the organism is grown. Much effort in recent years has gone into developing ways to produce microorganisms using various widely available materials as the substrates. Not surprisingly, enzymes produced by microorganisms have been used successfully for decades in food and food preparation.

2. NATURALLY OCCURRING MICROORGANISMS USED TO PRODUCE FOOD OR FOOD INGREDIENTS

One must assume that microorganisms grew in the foods of early humans and produced undesirable changes, which we now regard as spoilage. Some time later in the course of history, humans learned to use microorganisms deliberately to produce desirable changes in food.

No doubt our ancestors recognized that cooked meat spoiled less readily than raw meat. By adding salt to shredded cabbage they were able to produce sauerkraut. Adding salt to chopped meat produced a zesty tangy sausage, not a stinking slimy mess. By holding cucumbers in salt brine they obtained firm and tasty pickles. The same was true for green olives. Milk became sour and separated into whey and curd, the forerunner of cheese. Grape juice underwent spontaneous alcoholic fermentation, and if the product were held long enough it changed to vinegar. All of this was known long before we had heard about microbes. Humans simply learned by intuition and accident how to select for growth of certain types of microorganisms and produce desirable changes while inhibiting growth of unwanted types.

Pasteur's disproof of the theory of abiogenesis and his unequivocal demonstration of microorganisms as a leading cause of disease and the primary agent of decomposi-

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Food

Bread
Sourdough brea
Beer and ale
Wine
Vinegar

Soy sauce

Sauerkraut; pick
green olives
Fermented saus
Cultured butter
butter
Yogurt

Bulgarian butter
Acidophilus mil
Cheeses

Cottage, Crea
Neufchâtel
Cheddar, Eda
Swiss

Blue, Roquef
Brick, Limbu

Camembert

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TABLE 18

TRADITIONAL AMERICAN FERMENTED FOODS AND THE ORGANISMS USED IN
THEIR PRODUCTION IN THE UNITED STATES BEFORE 1958

Food	Microorganisms	See note
Bread	<i>Saccharomyces cerevisiae</i>	
Sourdough bread	<i>S. cerevisiae</i> plus various lactic acid-forming bacteria	4
Beer and ale	<i>S. cerevisiae</i> or <i>Saccharomyces carlsbergensis</i>	4
Wine	<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	5
Vinegar	<i>S. cerevisiae</i> var. <i>ellipsoideus</i> plus various acetic acid-forming species of <i>Acetobacter</i> or <i>Bacterium</i>	5
Soy sauce	<i>Aspergillus oryzae</i> plus various salt-tolerant yeasts and lactic acid bacteria	5
Sauerkraut; pickles and green olives	<i>Leuconostoc mesenteroides</i> , <i>Lactobacillus brevis</i> , and <i>Lactobacillus plantarum</i>	1, 5
Fermented sausage	Various lactobacilli; <i>Pediococcus cerevisiae</i>	4
Cultured buttermilk; butter	<i>Streptococcus cremoris</i> or <i>Streptococcus lactis</i> and <i>Leuconostoc dextranicum</i> or <i>L. citrovorum</i>	2, 4
Yogurt	<i>Streptococcus thermophilus</i> and <i>Lactobacillus bulgaricus</i>	3, 4
Bulgarian buttermilk	<i>L. bulgaricus</i>	3, 4
Acidophilus milk	<i>Lactobacillus acidophilus</i>	3, 4
Cheeses		3, 4
Cottage, Cream, Neufchâtel	<i>S. cremoris</i> or <i>S. lactis</i> and <i>L. dextranicum</i> or <i>L. citrovorum</i>	
Cheddar, Edam, Gouda	<i>S. cremoris</i> or <i>S. lactis</i>	3, 4
Swiss	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>L. lactis</i> , or <i>L. helveticus</i> ; and <i>Propionibacterium shermanii</i>	3, 4
Blue, Roquefort, Stilton	<i>S. lactis</i> or <i>S. cremoris</i> and <i>Penicillium roqueforti</i>	3, 4
Brick, Limburger	<i>S. lactis</i> or <i>S. thermophilus</i> , <i>Mycoderma</i> , <i>Geotrichum</i> spp., and <i>Bacterium linens</i>	3, 4
Camembert	<i>S. lactis</i> or <i>S. cremoris</i> , <i>Mycoderma</i> , <i>Geotrichum</i> spp., and <i>Penicillium camemberti</i>	3, 4

Notes

1. One large producer of fermented soy sauce has identified the organisms used as *Aspergillus oryzae* or *Aspergillus sojae*; *Pediococcus halophilus*; *Saccharomyces rouxii*; and *Candida* (*Torulopsis*) *versatilis* and *Candida etchellsii* (Sugiyama, 1984).
2. Various lactic acid bacteria are now available commercially for this purpose.
3. Now called *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*.
4. The microorganisms become an integral part of the food.
5. The microorganisms grow and produce their typical changes but are removed in whole or in part by centrifugation, filtration, or washing before the food is consumed. Thus, in usual circumstances, only their soluble products are consumed with the food.

tion of organic material led eventually to extensive studies of the organisms responsible for food fermentations. This made it possible to isolate and identify the desirable microbes and to add them deliberately as starter cultures. Using known organisms greatly decreases the likelihood of aberrant fermentations and ensures better quality products.

Table 18 lists many of our traditional fermented foods and the organisms used in their production (Foster *et al.*, 1957; Frazier, 1958). The long history of use of these

organisms and the widespread consumption of these foods and beverages testify to their safety. These organisms meet the criterion of "common use in foods in the United States before 1958." They may therefore reasonably be "generally recognized as safe" (GRAS).

Table 19 lists foods, food ingredients and enzymes that were produced industrially by microorganisms in the United States before 1958 (with the exceptions in notes 2 and 3).

Fermented foods have been produced in Oriental countries for centuries. Table 20 gives a partial list of the better known products. Some of these (e.g., Shoyu) have become important articles of commerce in Europe and North America.

3. MICROORGANISMS ASSOCIATED WITH FOOD

The common occurrence of harmless microorganisms in food is discussed at some length in Chapter 2. The fact that a specific microorganism is recognized in the published scientific literature as a harmless common contaminant in foods is relevant to establishment of its safety for use as a source of food ingredients. For instance, in the preamble to a GRAS affirmation regulation (Food and Drug Administration, 1983) the GRAS status of an enzyme product of *Bacillus licheniformis* was partially based on published information establishing that *B. licheniformis* is widely recognized as a harmless contaminant found in many foods.

4. MUTAGENESIS AND SELECTION OF MICROORGANISMS USED TO PRODUCE FOOD AND FOOD INGREDIENTS

Mutagenesis and selection techniques were first widely used in the 1940s with strains of *Penicillium* for the improvement of antibiotic production (Jacobson, 1981; Elander, 1982). In the intervening years remarkable improvements have been achieved using this technique in numerous other microorganisms of industrial importance including those used in the production of food ingredients such as citric acid, tryptophan, lysine, glutamic acid (Jacobson, 1981), and enzymes (Aunstrup *et al.*, 1979).

Mutations occur spontaneously in microbial populations; however, the observed frequency of a particular spontaneous mutation is usually lower than 10^{-5} . One would therefore have to examine as many as 100,000 colonies to observe a single mutation. Where a new phenotype can be selected for (such as growth on starch for an amylase positive mutant) even very infrequent spontaneous mutations can be detected easily. Frequently, however, it is not possible to select for a particular phenotype, and cells must be screened using various screening assays. These screening assays are often linked to computer analysis and automated methodology to screen large populations.

The proportion of mutants in a bacterial population can be increased by using mutagens—physical (e.g., ultraviolet irradiation), chemical (e.g., hydroxylamine, nitrosoguanidine), or biological (e.g., phage MU-1) agents. Some induce primarily base substitutions, others are efficient deletion mutagens, whereas still others can cause frameshifts (Jacobson, 1981).

The dose of the mutagen can alter the degree of mutation (Elander and Chang, 1979). Heavy doses can produce major changes in the morphology or biochemistry

EXAMPLES OF FOODS,

Product

Microorganisms themselves

Fats

Vitamins

Dextran
Lactic acid

Citric acid
Enzymes
Amylases

Invertase
Pectinases

Proteases

Glucose oxidase

1. Yeasts are often consumed as the brewing industry or they are used as a fermentable carbohydrate are available during wartime.
2. The mold *Geotrichum candidum* is used for the production of citric acid.
3. These organisms were used in the production of vitamins.
4. Used primarily for the vitamins.
5. Used for fat-soluble vitamins.

of the organism. Small doses of the organism. Sequential mutagenesis successfully in yield improvement. Mutagenesis and selection require an extensive knowledge

TABLE 19

EXAMPLES OF FOODS, FOOD INGREDIENTS, AND ENZYMES PRODUCED INDUSTRIALLY BY
MICROORGANISMS PRIOR TO 1958

Product	Microorganisms	See note
Microorganisms themselves	<i>Saccharomyces cerevisiae</i>	1
	<i>Saccharomyces carlsbergensis</i>	1
	<i>Geotrichum candidum</i>	2
	<i>Cryptococcus (Torulopsis) utilis</i>	1
	<i>Candida arborea</i>	1
	<i>Torula pulcherrima</i>	1
Fats	<i>Torulopsis pulcherrima</i>	3
	<i>Geotrichum candidum</i>	3
	<i>Endomyces vernalis</i>	3
	<i>S. carlsbergensis</i>	5
Vitamins	<i>Aspergillus fisheri</i>	5
	<i>Clostridium acetobutylicum</i>	4
	<i>Eremothecium ashbyii</i>	4
	<i>Ashbya gossypii</i>	4
	<i>Streptomyces</i> spp.	4
	<i>Leuconostoc mesenteroides</i>	
Dextran	<i>Lactobacillus delbrueckii</i>	
Lactic acid	<i>Lactobacillus bulgaricus</i>	
	<i>Lactobacillus plantarum</i>	
	<i>Bacillus coagulans</i>	
	<i>Aspergillus niger</i>	
Citric acid		
Enzymes		
	<i>Aspergillus oryzae</i>	
	<i>Rhizopus delemar</i>	
	<i>Mucor rouxii</i>	
	<i>Bacillus subtilis</i>	
Invertase	<i>S. cerevisiae</i>	
Pectinases	<i>Aspergillus</i> spp.	
	<i>Penicillium</i> spp.	
Proteases	<i>Aspergillus oryzae</i>	
	<i>Bacillus subtilis</i>	
Glucose oxidase	<i>Aspergillus niger</i>	

Notes

1. Yeasts are often consumed as sources of protein or vitamins. They may be obtained as by-products of the brewing industry or they may be produced directly for food use when inexpensive sources of fermentable carbohydrate are available.
2. The mold *Geotrichum candidum* has been used in some countries as a source of protein and vitamins during wartime.
3. These organisms were used in Germany and Sweden as sources of fat during World Wars I and II.
4. Used primarily for the vitamins of the B complex.
5. Used for fat-soluble vitamins.

of the organism. Small doses can result in subtle changes in the phenotype of an organism. Sequential mutagenesis with small doses of mutagens has been used successfully in yield improvement programs (Elander and Chang, 1979).

Mutagenesis and selection constitute a random process and do not necessarily require an extensive knowledge of the genetics of the microorganism to be successful.

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tries for centuries. Table 20 of these (e.g., Shoyu) have orth America.

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lations; however, the observed usually lower than 10^{-5} . One 00 colonies to observe a single r (such as growth on starch for spontaneous mutations can be to select for a particular pheno- ing assays. These screening as- omated methodology to screen

tion can be increased by using emical (e.g., hydroxylamine, ni- nts. Some induce primarily base s, whereas still others can cause

mutation (Elander and Chang, the morphology or biochemistry

TABLE 20
SOME ORIENTAL FOODS PRODUCED BY MICROBIAL ACTION

Nature of food product	Microorganisms	Substrate	
Tempeh	<i>Rhizopus</i> sp.	Soybeans	Solid
Sufu	<i>Actinomucor elegans</i> , <i>Mucor</i> sp.	Soybeans	Solid
Ragi	<i>Mucor</i> sp., <i>Rhizopus</i> sp., yeast	Rice	Solid
Tea fungus	<i>Acetobacter</i> sp., two yeasts	Tea extract and sucrose	Liquid
Miso	<i>Aspergillus oryzae</i> , <i>Saccharomyces rouxii</i>	Rice and other cereals	Paste
Shoyu	<i>Aspergillus oryzae</i> , <i>Lactobacilli</i> , <i>Hansenula</i> sp., <i>Saccaromyces</i> sp.	Soybeans and wheat	Liquid
Ang-kak (red rice)	<i>Monascus purpurea</i>	Rice	Solid
Natto	<i>Bacillus subtilis</i>	Soybeans	Solid
Nata	<i>Acetobacter</i> sp.	Fruit juices	Gel

Source. Adapted from Hesseltine (1965).

These have been used extensively to optimize strain properties such as development of a constitutive mutant that does not require an expensive or undesirable inducer and elimination of objectionable by-products such as antibiotics or undesirable enzymatic side activities (Aunstrup *et al.*, 1979).

There is little doubt that genetic modification of producer strains by mutagenesis coupled with rational selection procedures has been the most important single factor contributing to the success of the fermentation industry in producing food ingredients, pharmaceuticals, industrial enzymes, and other chemicals. In the future it is anticipated that the ability to move well-defined genes from a large number of donor microorganisms into a relatively small number of genetically well-studied host organisms will lead to a better understanding of the complex cellular regulatory control that has been modified to yield higher production in improved mutants (Elander, 1982). This will lead to an increasingly rapid development of the use of microorganisms to produce useful products, including food products.

5. EVALUATION OF FOOD INGREDIENTS DERIVED FROM GENETICALLY MODIFIED MICROORGANISMS

Recently, the advent of biotechnology has given us the ability to use microbes and enzymes in new and better ways. For example, cheesemaking has traditionally relied on the enzyme rennin, prepared from calf stomach. Biotechnology has enabled the efficient preparation of this same enzyme from microbes engineered with the rennin-encoding gene.

According to a National Academy of Sciences (1987) report there is no evidence of a unique hazard from the transfer of genes between organisms. Nonetheless food and food ingredient manufacturers and suppliers, and the federal agencies responsible for food safety regulation, are committed to ensuring the public that the products

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FIG. 5. Decision tree for evaluating relative safety of food ingredients derived from genetically modified microorganisms.

	If Yes	If No
	Proceed to	
1. Does the microbe end up in food?	2	4
2. Is the organism free of transferable antibiotic resistance genes? (see Appendix A)	4	3
3. Does the resistance gene code for resistance to a substance used in control of disease agents in human or veterinary medicine?	Table 21, part D	4
4. Are the vectors characterized and free of attributes that would render them unsafe for constructing microorganisms to be used to produce food-grade products? (see Appendix B)	5	Table 21, part D
5. Does the DNA insert code for a substance safe for use in food? (see Appendix C)	6	Table 21, part D
6. Is the microbe free of DNA from an intermediate host which could code for a toxic product? (see Appendix D)	Table 21, part A	Table 21, part D

of biotechnology are safe for consumption. The decision tree developed in this document is modeled after an earlier one developed by Pariza and Foster (1983). It has been widely accepted by the scientific community for determining safety assessment criteria for microbial enzyme preparations used in food. The Pariza and Foster approach has been extended in this section to cover food ingredient products obtained from genetically modified microorganisms.

6. DECISION TREE FOR EVALUATING RELATIVE SAFETY OF FOOD INGREDIENTS DERIVED FROM GENETICALLY MODIFIED MICROORGANISMS

The focus of the decision tree is on the safety of the organism and the products it produces. It is assumed that if the organism is nontoxigenic and nonpathogenic, then foods or food ingredients produced from the organism under current Good Manufacturing Practices will be safe to consume. Whole foods produced from microorganisms can best be evaluated by using the decision tree in Chapter 6.

As currently developed, the decision tree (Fig. 5) extends the Pariza and Foster approach (Table 21) to genetically modified organisms and represents a conservative guide to safety evaluation. No organism or product can be accepted without testing for toxin production, and in most cases this will involve animal studies. It is expected that the proposed scheme will evolve as the safety data base on new organisms from biotechnology expands.

A number of microorganisms such as some species of *Bacillus*, *Saccharomyces*, *Lactobacillus* and *Aspergillus* have a documented history of safe use in food. Thus, we regard the transfer of a gene from a nonpathogenic, nontoxigenic source to a similarly safe host, especially one that is already part of the food chain, as a safe

TABLE 21

GUIDELINES FOR DETERMINING THE SAFETY OF FOOD INGREDIENTS
DERIVED FROM MICROORGANISMS^a

	If yes Proceed to	If no
A. Decision tree		
1. Is the test material free of antibiotics? ^b	A.2	D
2. a. For bacteria and yeast:		
i. Is the test material free of toxins ^{c,d} known to be produced by other strains of the same species?	A.3	D
ii. If there are no known toxins ^{c,d} produced by other strains of the same species, is the no-observable-effect level (NOEL) in a single oral challenge sufficiently high to ensure safety? ^{e-g}	B	D
b. For molds, is the test material free of detectable levels of aflatoxin B ₁ , ochratoxin A, sterigmatocystin, T-2 toxin, zearalenone, and any other toxins known to be produced by strains of the same species? ^h	C	D
3. Is the NOEL in short-term feeding studies sufficiently high to ensure safety? ^{e-g}	ACCEPT	D
B. Special considerations for certain yeasts and bacteria:		
1. If the source culture is a well-known, widely distributed, nonpathogenic yeast, e.g., certain species of the genus <i>Saccharomyces</i> , or if it belongs to a bacterial species that is well characterized, commonly present in foods, has a history of safe use in food ingredient manufacture, and has never been implicated in foodborne disease, e.g., <i>Bacillus coagulans</i> , <i>Bacillus licheniformis</i> , <i>Micrococcus lysodeikticus</i> , and <i>Bacillus subtilis</i> (Buchanan and Gibbons, 1974), the test material can be <i>ACCEPTED</i> at this point.		
2. Test material from other bacteria and yeasts must be considered under part A.3.		
C. Special considerations for certain molds:		
1. If the source culture is well characterized, commonly present in food, has a history of safe use in food ingredient manufacture, and has never been implicated in foodborne intoxication or disease, e.g., <i>Aspergillus oryzae</i> , <i>Aspergillus niger</i> , and <i>Rhizopus oryzae</i> (Beckhorn <i>et al.</i> 1965; Fennel, 1976; Moskowitz and Cayle, 1974; Riemann and Bryan, 1979; Rogers, 1977; Roland, 1981; Scott, 1980; Stoloff <i>et al.</i> , 1977), the test material can be <i>ACCEPTED</i> at this point.		
2. Test material from all other species of molds must be considered under part A.3.		
D. Disposition of materials that fail any decision tree requirements: A negative answer to question 1, 2, or 3 signifies the presence of an undesirable substance and the material is not acceptable for use in food. If the undesirable substance can be removed, the purified material must be passed through the system again, beginning at the point of the original negative answer.		

Source. This table is essentially reproduced from Pariza and Foster (1983). See original source for further discussions and rationale.

^a These guidelines are intended for crude culture extracts, for whole cultures, and for concentrated enzyme or other microbially derived fractions which, when diluted, become preparations suitable for marketing.

^b As determined by (Anonymous, 1981) or comparable methods.

^c For the purposes of these guidelines, the term *toxin* refers to a substance which is regarded by experts as a cause of food poisoning, intoxication, or illness when ingested. Examples are staphylococcal enterotoxins, botulinal neurotoxins, and mycotoxins.

^d Certain cultures in this category are acceptable on the basis of single acute oral toxicity test, as explained in part B.1. Cultures that fall under part B.2 can go directly to part A.3 without an acute oral toxicity test. This is permissible because the subchronic feeding specified in part A.3 is more rigorous and more meaningful than the acute oral toxicity test embodied in part A.2.a.ii.

^e Expressed as mg/kg body wt and determined using appropriate animal species.

^f Estimated mean consumption level is calculated from the sum of the intakes for each food category in which the material is expected to be used. An example of such determination is (USDA mean portion size) × (Market Research Corporation of American eating frequency for the entire population) × (the usual level of use expressed as total organic solids (TOS) for microbial preparation in question) (Anonymous,

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system either for enzyme or ingredient production or for direct use in a food product. In some cases the vector used has also been determined to be safe on the basis of full sequencing and characterization. In these cases, the exact structure of the new genetic construct is known and should be considered safe. pBR322 and pUB110 are examples of such vectors (see Appendixes A and B).

In cases where an entire gene is deleted from a microbe in current use, usually additional safety testing may not be necessary. For instance, deletion of a sporulation gene from a *Bacillus* strain used for α -amylase production should not raise any safety issues about the α -amylase itself.

Mutations important in industrial yield improvement programs (Elander and Chang, 1979; Elander, 1982) are usually the result of the alteration of a regulatory gene for production of a given product or cellular function. It is not possible to convert an organism into a toxin producer by mutagenesis if it lacks the gene(s) for synthesizing the toxin in question. It is important to keep in mind, however, that under certain growth conditions, toxigenic strains may not express the toxin. Organisms that have a history of use in food processing are preferred. New microbial isolates should be evaluated under a variety of growth conditions for the ability to produce toxins elaborated by other strains in the same species. It is not possible to establish absolutely that a strain is nontoxigenic solely from data on toxin expression. Therefore, in cases where a new, less familiar host, vector, or gene is used we propose that the material be tested as suggested by Pariza and Foster (1983).

To date the Food and Drug Administration has accepted for filing six GRAS petitions (CPC International, Ltd., 1986; Enzyme Bio-Systems, Ltd., 1988; Pfizer, Inc., 1988a; Gist-Brocades, Inc., 1989; Genencor, Inc., 1989; Novo Laboratories, Inc., 1990) and one food additive petition (Pfizer, Inc., 1988b) concerning food ingredients derived from rDNA-modified microorganisms. In response to the Pfizer petitions (1988a, b), the regulations were recently amended (Food and Drug Administration, 1990) to affirm that the use of a chymosin preparation derived by fermentation from *E. coli* K-12 is generally recognized as safe (GRAS). The rest of the above petitions are currently under review by the Agency. In addition, a number of other GRAS petitions for products from genetically modified microorganisms have been submitted and are currently under prefilling review by the agency.

According to a paper prepared for the 18th session of the Codex Alimentarius Commission (Berkowitz and Maryanski, 1989), there is no evidence of unique hazards associated with rDNA technology and that potential risks which may occur are the same kind as those associated with conventional methods. Safety evaluation should be based on accumulated experience and scientific knowledge of the characteristics of the finished food substance.

1972, 1982). TOS is defined as the sum of the organic compounds, excluding diluents, contained in the final microbial preparation (Pariza and Foster, 1983).

^a The term *sufficiently high* refers to appropriate multiples of the estimated mean human consumption level. Where the product is an incidental additive or processing aid (e.g., an enzyme) the NOEL should be at least 100 times the estimated mean human consumption level. Where the product is itself a food (e.g., yogurt) or a major food component (e.g., mycoprotein) it may not be possible to test at this high a level. In these cases, safety may be established by feeding the highest level compatible with the maintenance of adequate nutritional requirements and consideration of the questions outlined in the decision tree for whole foods and complex mixtures (Fig. 7).

^b As determined by Patterson and Roberts (1979) or comparable methods.

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With regard to the safety evaluation of improved production microorganisms to produce substances that are already marketed, Berkowitz and Maryanski stated that the safety evaluation should focus on the following factors:

- (i) the identity of the host organism;
- (ii) any evidence of pathogenicity or toxin production;
- (iii) the function of the inserted gene(s);
- (iv) the identity of organisms that contribute genetic material to the final construct;
- (v) characterization of the inserted genetic material to ensure the absence of sequences that may encode harmful substances;
- (vi) insertional and genomic stability;
- (vii) chemical specifications;
- (viii) dietary use and exposure and other relevant information.

The IFBC agrees that these criteria are relevant to the safety evaluation of such microorganisms.

7. APPENDIXES

Appendix A. Antibiotic Resistance Genes

Is the organism free of transferable antibiotic resistance genes?

Antibiotic resistance genes, often originally from transposons, are integral parts of most common vectors. These marker genes allow cells transformed with the vector to be distinguished from nontransformed cells. Many of these resistance genes, especially those of therapeutic importance, were originally isolated from plasmids.

The use of antibiotic resistance genes as selectable markers in microorganisms has been questioned since antibiotic resistance is common in bacteria that cause disease in humans and animals and is usually determined by plasmids (Saunders, 1984). The prevalence of such plasmids and the range of drugs to which they confer resistance have increased greatly in the past 30 years (Hughes and Datta, 1983). The mechanisms (conjugation, transformation, and transduction) by which bacteria exchange genes have been reviewed (Saunders, 1984). The human bacterial flora had the potential to transfer genes long before resistance became a problem (Hughes and Datta, 1983; Saunders, 1984). The reported incidence of bacteria that harbor plasmids conferring resistance is normally higher in countries where the use of antibiotics is not controlled, and in hospitals as compared to the community at large (Falkow, 1975; Saunders, 1984). The proportion of strains resistant to specific drugs can also be related to changes in antibiotic policy within hospitals (Buckwold and Ronald, 1979; Saunders, 1984). These findings strongly suggest that there is a causal relationship between antibiotic use (and overuse) and the evolution of a resistant bacterial flora (Saunders, 1984). The preceding strongly indicates that the development of antibiotic resistance among bacterial populations is not due to the availability of plasmids, but rather is the genetic consequence of imposing selective pressure on these populations by the introduction of therapeutic antibiotics into clinical use.

Cloning vectors containing resistance genes as selectable markers are usually constructed such that the resistance genes are no longer transposable. The resistance genes on such vectors can be considered to be stably associated with the vector.

If the rDNA organism does not enter the food product or if the organism is not deliberately released to the environment, then the presence of antibiotic resistance

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genes should also not be a concern. This is because the expression products of such genes do not add toxic components to the food supply and, more importantly, the genes themselves will not be transferred to other organisms. In many cases the recombinant microorganism is used in a contained fermentation facility to produce an enzyme or other food ingredient. The recombinant microorganism is then removed from the commercial product. The residual microbial biomass is treated so as to inactivate the production microorganism before it is disposed of by spreading on agricultural land, in sanitary landfills, or other appropriate means. The small numbers of recombinant microorganisms that may enter the environment under these conditions should be of no consequence (National Academy of Sciences, 1987).

In cases where the microorganism does enter the food product or will be released directly to the environment, then the presence of antibiotic resistance genes may be a concern. In such cases the extent to which the presence of the genes will compromise the use of antibiotics to control disease agents in human or veterinary medicine must be evaluated. This is considered further in Appendix B.

Appendix B. Characterization of Vectors

Are the vectors characterized and determined to be safe for genetically modifying microorganisms to be used to produce food-grade products?

The key issue is the gene product itself and its safety in food applications. The vector will have no negative safety impact on the final product unless (1) it produces toxic substances that are seen in the final product; (2) it affects the production of toxic substances by the host production strain that are seen in the final product; or (3) it contains a mobile antibiotic resistance gene that could ultimately be transferred from the production strain to pathogens in the intestinal microflora. In cases where the production strain does not contact humans, animals, or other microorganisms, minimum safety concerns should exist with regard to the vector.

We would set a standard for a safe plasmid as one which after extensive use and testing in microbial systems is not known to generate any toxic material, or one for which there is extensive evidence not to expect toxin to be generated. This would include, but not be limited to (1) plasmids with documented prior safe use in the preparation of a food product [thus far, this includes pBR322 and pUB110 used and evaluated in food enzyme production (Pfizer, Inc., 1988a; MacKenzie *et al.*, 1989a,b; Andersen *et al.*, 1987; Diderichsen and Christiansen, 1988; U.S. Food and Drug Administration, 1990)] and (2) plasmids whose complete DNA sequence is known and which have also been shown not to encode any protein toxin found in a species with which the plasmid is associated.

A well-characterized plasmid, one whose full DNA sequence is known and whose genes have been defined, should be the vector of choice. Currently, the best known plasmid is pBR322 which has been reviewed by Balbas *et al.* (1986). Plasmid pUB110 has also been characterized at this level (McKenzie *et al.*, 1986, 1987); several other yeast and *Aspergillus* plasmids have been characterized, but not as well as pBR322 and pUB110.

It should be possible either to use a plasmid derived from a nonpathogenic, nontoxigenic strain or to show that toxins produced by the strain from which the plasmid is obtained are not encoded by the plasmid. Hence, in the case of pUB110, obtained from *Staphylococcus aureus*, genes for several of the well known enterotoxins such

as A, B, and C have been cloned and sequenced, and it can be shown that pUB110 does not encode for any of these.

It should be noted, however, that knowledge of the DNA sequence of a plasmid cloning vector is not an assurance of safety. For example, the sequence of pBR322 has been corrected at least twice since its initial publication, and that of pUB110 at least once. The consequence of the corrections is that new potential reading frames to encode proteins are constantly being revised, and the assurances of today become tomorrow's questions. A second problem is that even given an apparently safe DNA sequence, a potential open reading frame may be difficult to correlate with a function. For example, authors still disagree over the nature of the actual product encoded by the pUB110 *alpha* gene as well as where the gene actually starts. However, when the protein sequence of a toxin or the DNA sequence of its gene is known, it can be stated with assurance that toxin production is not determined by a given plasmid (for example, there are no similarities between the sequence of pUB110 and the DNA sequence of the *Staphylococcus aureus* toxin B). While knowledge of the DNA sequence of a plasmid or construct represents a significant step in our understanding of its function, such information only increases the comfort level with which we can use the plasmid, and does not, by itself, provide absolute assurance of safety.

A partial list of plasmids certified for use in cloning experiments may be found in the NIH Guidelines (*Fed. Reg.* 51, 16970-16971). The most complete list of available plasmids may be found in the series *Cloning Vectors* (Pouwels *et al.*, 1985 and supplements in 1986 and 1987); however, many more plasmids have become available since the 1987 list was assembled.

Other aspects related to the safety of a vector used in rDNA technology are (1) whether the strain carries genetically modified extrachromosomal DNA and (2) whether the gene of interest has been integrated into the chromosome.

I. In strains with extrachromosomal DNA one should consider two factors:

- A. The presence or absence of relevant human or animal antibiotic resistance marker genes. The concern is the possibility of compromising medical or veterinary antibiotic therapy if the antibiotic resistance gene is transferred to pathogenic intestinal microflora.
- B. The possibility that extrachromosomal DNA might increase the overall toxicity of the final product by the action of proteins produced from other coding regions.

To avoid these problems one has three options:

1. Take the extrachromosomal DNA from a microorganism that is known to be safe in food applications.
 2. Use extrachromosomal DNA that is itself known to be safe (e.g., pUB110 or pBR322).
 3. Use a vector that has been sufficiently characterized to determine the presence of other functional genes, if any, and the lack of toxicity of the gene's products (restriction analysis, Northern analysis, sequencing).
- II. In strains with the gene of interest integrated into the chromosome one needs to consider three factors:
- A. Mobility of the insert within the chromosome and movement to extrachromosomal DNA with subsequent transfer to intestinal pathogens. This refers to the use of mobile transposons, which are short sections of double-stranded DNA that consist of more than 2000 base pairs. They are able to move within the genome, even between a chromosome and a plasmid transferring genes

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relevant to the treatment of human or animal diseases. It is also possible, if a strain carries plasmids which have regions of homology with inserted DNA, that the gene could be transferred from the chromosome to a free plasmid by homologous recombination. The plasmid would need to be transferable and able to move by itself (self-mobilizable) for exchange to other organisms to be possible.

- B. The nature of the genetic insert. This involves the presence of the gene of interest and any supporting DNA spacers, linkers, etc., and vector DNA.
- C. The location of the insert, which may inactivate genes.

To resolve these issues one may do the following:

1. Inactivate the mobility of transposons, if used.
2. Eliminate the possibility that mobilizable plasmids are present which could "rescue" the inserted DNA from the chromosome.
3. Eliminate the possibility of transferring antibiotic resistance genes to the intestinal microflora.
4. Use homologous recombination for gene insertion.
5. Insert the gene of interest at the same site as the wild type or any other gene which in its absence does not affect the toxicity of the final product.

The Food and Drug Administration (1990) concluded that chymosin preparation from a recombinant strain of *E. coli* K-12 made in conformity with 21CFR § 184.1685 will not contain DNA encoding resistance to antibiotics at levels that would provide any safety concern. This conclusion was based on a gel electrophoresis/DNA hybridization experiment and a transformation assay submitted by Pfizer, Inc. (1988b) demonstrating that the enzyme preparation does not contain gene-size DNA fragments or transformable DNA. In the electrophoresis experiment, DNA fragments were sized on the basis of their differential rates of migration through the gel and quantitated on the basis of their level of hybridization with labeled complementary DNA. No DNA fragments large enough to contain an intact gene encoding antibiotic resistance were detected in the enzyme preparation.

In the transformation assay, bacterial cells were mixed with DNA under optimized conditions to see if they had picked up the antibiotic resistance encoded by the DNA. Cells mixed with the enzyme preparation did not become antibiotic resistant.

Appendix C. Safety of DNA Insert

Does the DNA insert code for a substance safe for use in food?

Safety evaluation should focus on the organism that embodies the final construct. The nature of the gene donor should not be of particular importance except as it may guide the assessment of safety of the final construct. For example, any toxic potential of the gene source organism should be addressed in the safety evaluation scheme.

Two considerations should guide safety evaluation of the DNA insert. First, it should be shown that the insert itself is safe; second, it should be shown that use of the insert does not produce a pleiotropic effect (secondary phenotypic alteration resulting from a single genetic change) (Tiedje *et al.*, 1989) that results in elaboration of a toxin.

The DNA insert is important in that it codes for a desirable product. Safety evaluation of the insert should focus on its expression product.

The possibility of a pleiotropic effect resulting in toxicity is greatly diminished by using a host organism that does not produce toxins. For prokaryotes a demonstration of nontoxicity is fairly easily accomplished because of the relative simplicity of the genome (Pariza and Foster, 1983). However, for eukaryotic microorganisms (especially molds) such a demonstration may be more difficult. There are many examples where potentially toxic products are elaborated by eukaryotes only under special conditions (Pariza and Foster, 1983). At other times, toxin is not produced. The products of the construct intended for use in food should therefore be tested for toxicity under the exact conditions that will be used for routine growth in the manufacturing plant. Toxicity should be evaluated using chemical tests for specific toxins as well as animal assays (decision tree, Fig. 5) (Pariza and Foster, 1983).

Appendix D. DNA from Intermediate Hosts

Is the microbe free of DNA from an intermediate host which could code for a toxic product?

Recombinant DNA procedures usually rely on an initial cloning of the gene of interest in what is termed an intermediate host. Due to extensive genetic knowledge and 40 years of laboratory experience with the organism, *Escherichia coli* is the most common (though certainly not the only possible) intermediate host. During construction of the recombinant vector, it is technically possible that small portions of the intermediate host DNA may be transferred along with the vector and the cloned gene. If the intermediate host is a nontoxigenic, nonpathogenic organism, it is not possible that these pieces (regardless of size) will render the production organism toxic. When the intermediate host is known to carry toxin genes, then it becomes imperative to show that any intermediate host DNA in the final construction does not code for a toxin. This proof could be based on an evaluation of the DNA sequence if the toxin has been cloned and its sequence is known. Alternatively, classical methods for showing lack of toxicity in the final product should be sufficient.

In cases where the intermediate DNA constitutes regulatory regions (i.e., promoters, terminators) which are themselves not expressed, no further testing would be necessary. Usually these regulatory regions are selected for use by design and have been completely sequenced, and it is clear that they do not code for proteins. If long regions which might potentially code for proteins are used, they could be confirmed to be nonfunctional by (1) lack of promoter regions upstream or (2) lack of mRNA complementary to the DNA.

REFERENCES

- ANDERSEN, J. R., DIDERICHSEN, B. K., HJORTKJAER, R. K., DEBOER, A. S., BOOTMAN, J., WEST, H., AND ASHBY, R. (1987). Determining the safety of maltogenic amylase produced by rDNA technology. *J. Food Prot.* **50**, 521-526.
- Anonymous (1972). A comprehensive survey of industry on the use of food chemicals generally recognized as safe (GRAS). *Comprehensive GRAS Survey*. National Academy of Sciences/National Research Council, U.S. Department of Commerce, Springfield, VA. NTIS PB221-949.
- Anonymous (1981). *Specification for Identity and Purity of Carrier Solvent, Emulsifiers and Stabilizers, Enzyme Preparations, Flavoring Agents, Food colours, Sweetening Agents and Other Food Additives*. Food and Nutrition paper No. 19, pp. 215-218. 25th session of the Joint Expert Committee on Food Additives, FAO/WHO, Rome.

- Anonymous (1982). U.S.A. comment to the ad hoc working group on food additive intake. 15th Session, Codex Committee of Food Additives, Joint FAO/WHO. Food Standards Programme, Hague.
- AUNSTRUP, K., ANDRESEN, O., FALCH, E. A., AND NIELSEN, T. K. (1979). Production of microbial enzymes. In *Microbial Technology*, Vol. 1, 2nd ed., *Microbial Processes* (H. J. Peppler and D. Perlman, Eds.), pp. 281–309. Academic Press, New York.
- BALBAS, P., SOBERON, X., VALLE, F., MERINO, E., ZURITA, M., LOMELI, H., FLORES, N., AND AOLIVAR, F. (1987). Plasmid vector pBR322 and its special purpose derivatives—A review. *Gene* 50, 3–40.
- BECKHORN, E. J., LABBE, M. D., AND UNDERKOFER, L. A. (1965). Production and use of microbial enzymes for food processing. *J. Agric. Food Chem.* 13, 30–34.
- BERKOWITZ, D., AND MARYANSKI, J. (1989). Implications of biotechnology on international food standards and codes of practice. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, Eighteenth Session, Geneva, July 3–12.
- BUCHANAN, R. E., AND GIBBONS, N. E. (Eds.) (1974). *Bergey's Manual of Determinative Bacteriology*, 8th ed. Williams & Wilkins, Baltimore, MD.
- BUCKWOLD, F. J., AND RONALD, A. R. (1979). Antimicrobial misuse—Effects and suggestions for control. *J. Antimicrob. Chemother.* 5, 129–136.
- CPC International, Ltd. (1986). GRAS Petition 4GO293 proposing that *alpha*-amylase from *B. subtilis* (ATCC 39,705) containing a gene for *alpha*-amylase from *B. stearothermophilus* inserted by recombinant DNA techniques be affirmed as GRAS as a direct human food ingredient. Notice of Filing. *Fed. Regist.* 51, 10571.
- DIDERICHSEN, B. K., AND CHRISTIANSEN, L. (1988). Cloning of a maltogenic *alpha*-amylase from *Bacillus stearothermophilus*. *FEMS Microbiol. Lett.* 56, 53–60.
- ELANDER, R. P. (1982). Traditional versus current approaches to the genetic improvement of microbial strains. In *Overproduction of Microbial Products* (V. Krumphanzl, B. Sikyta, and Z. Vanik, Eds.), pp. 353–369. Academic Press, New York.
- ELANDER, R. P., AND CHANG, L. T. (1979). Microbial culture selection. In *Microbial Technology*, Vol. 2, 2nd ed., *Fermentation Technology* (H. J. Peppler and D. Perlman, Eds.), pp. 243–302. Academic Press, New York.
- Enzyme Bio-Systems, Ltd. (1988). GRAS Petition 7GO328 proposing that *alpha*-amylase produced from a strain of *Bacillus subtilis* containing a *Bacillus megaterium alpha*-amylase gene be affirmed as GRAS as a direct human food ingredient. Notice of Filing. *Fed. Regist.* 53, 16191.
- FALKOW, S. (1975). *Infectious Multiple Drug Resistance*. Pion, London.
- FENNEL, D. I. (1976) *Aspergillus oryzae* (NRRL Strain 1988): A clarification. *Science* 194, 1188.
- Food and Drug Administration (1983). Direct food substances affirmed as GRAS: Mixed carbohydrase and protease enzyme product. *Fed. Regist.* 48, 239–240.
- Food and Drug Administration (1990). Direct food substances affirmed as GRAS: Chymosin enzyme preparation derived from *Escherichia coli* K-12. *Fed. Regist.* 55, 10932–10935.
- FOSTER, E. M., NELSON, F. E., SPECK, M. L., DOETSCH, R. N., AND OLSON, J. C., JR. (1957). *Dairy Microbiology*. Prentice-Hall, Englewood Cliffs, NJ.
- FRAZIER, W. C. (1958). *Food Microbiology*. McGraw-Hill, New York.
- Genencor, Inc. (1989). GRAS Petition 9GO352 proposing that chymosin derived from a genetically modified *Aspergillus niger* var. *awamori* be affirmed as GRAS as a direct human food ingredient. Notice of Filing. *Fed. Regist.* 54, 40910–40911.
- Gist-Brocades, Inc. (1989). GRAS Petition 9GO349 proposing that chymosin derived from a genetically modified *Kluyveromyces marxianus* var. *lactis* be affirmed as GRAS as a direct human food ingredient. Notice of Filing. *Fed. Regist.* 54, 20203.
- HELSELTINE, C. W. (1965). A millenium of fungi, food and fermentation. *Mycologia* 57, 149–197.
- HUGHES, V. M., AND DATTA, N. (1983). Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature (London)* 302, 725–726.
- JACOBSON, G. K. (1981). Mutations. In *Biotechnology—A Comprehensive Treatise in 8 Volumes*. Vol. 1. *Microbial Fundamentals* (H.-J. Rehm and G. Reed, Eds.), pp. 279–304. Verlag Chemie, Deerfield Beach, FL.
- MACKENZIE, K. M., PETSEL, S. R. W., WELTMAN, R. H., AND ZEMAN, N. W. (1989a). Subchronic toxicity studies in dogs and *in-utero*—Exposed rats fed diets containing *Bacillus stearothermophilus alpha*-amylase from a natural or recombinant DNA host. *Food Chem. Toxicol.* 27, 599–606.
- MACKENZIE, K. M., PETSEL, S. R. W., WELTMAN, R. H., AND ZEMAN, N. W. (1989b). Subchronic toxicity

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- MCKENZIE, T., HOSHINO, T., TANAKA, T., AND SUBOKA, N. (1986). The nucleotide sequence of pUB110: Some salient features in relation to replication and its regulation. *Plasmid* **15**, 93–106.
- MCKENZIE, T., HOSHINO, T., TANAKA, T., AND SUBOKA, N. (1987). A revision of the nucleotide sequence and functional map of pUB110. *Plasmid* **17**, 83–85.
- MOSKOWITZ, G. J., AND CAYLE, T. (1974). A method for detection of beta-nitropropionic acid in crude biological extracts. *Cereal Chem.* **51**, 96–105.
- National Academy of Sciences, Committee on the Introduction of Genetically Engineered Organisms into the Environment (1987). *Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issues*. National Academy Press, Washington, DC.
- Novo Laboratories, Inc. (1990). GRAS Petition 7GO326 proposing that maltogenic amylase enzyme preparation derived from a genetically modified *Bacillus subtilis* be affirmed as GRAS as a direct human food ingredient. Notice of Filing. *Fed. Regist.* **55**, 9772–9773.
- PARIZA, M. W., AND FOSTER, E. M. (1983). Determining the safety of enzymes used in food processing. *J. Food Prot.* **46**, 453–468.
- PATTERSON, D. S., AND ROBERTS, B. A. (1979). Mycotoxins in animal feedstuffs: Sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone, and T-2 toxin. *J. Assoc. Off. Anal. Chem.* **62**, 1265–1267.
- Pfizer, Inc. (1988a). Food Additive Petition 8A4048 proposing amendment of the food additive regulations to provide for the safe use of a genetically modified *E. coli* K-12 as a source of chymosin for use in food. Notice of Filing. *Fed. Regist.* **53**, 3792.
- Pfizer, Inc. (1988b). GRAS Petition 8GO337 proposing that chymosin derived from the fermentation of a genetically modified *E. coli* K-12 be affirmed as GRAS as a direct human food ingredient. Notice of Filing. *Fed. Regist.* **53**, 3792.
- POUWELS, P. H., ENGER-VALK, B. E., AND BRAMMAR, W. J. (1985; 1986 and 1987 updates). *Cloning vectors, a Laboratory Manual*. Elsevier, New York.
- RIEMANN, H., AND BRYAN, F. L. (Eds.) (1979). *Food-borne Infections and Intoxications*, 2nd ed. Academic Press, New York.
- ROGERS, R. W. (1977). *Monograph on Microbial Enzymes*, National Technical Information Service Document PB-289-661. U.S. Department of Commerce, Springfield, VA.
- ROLAND, J. F. (1981). Regulation of food enzymes. *Enzyme Microb. Technol.* **3**, 105–110.
- SAUNDERS, J. R. (1984). Genetics and evolution of antibiotic resistance. *Brit. Med. Bull.* **40**, 54–60.
- SCOTT, D. (1980). Enzymes, industrial. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed., Vol. 9 (M. Grayson and D. Eckroth, Eds.), pp. 173–224. Wiley, New York.
- STOLOFF, L., MISLIVEC, P., AND SCHINDLER, A. F. (1977). *Aspergillus oryzae* (NRRL strain 1988). *Science* **196**, 1354.
- Subcommittee on Microbiological Criteria for Foods and Food Ingredients, National Research Council (1985). *An Evaluation of the Role of Microbiological Criteria for Foods and Food Ingredients*. National Academy Press, Washington, DC.
- SUGIYAMA, S.-I. (1984). Selection of microorganisms for use in the fermentation of soy sauce. *Food Microbiol.* **1**, 339–347.
- TIEDJE, J. M., COLWELL, R. K., GROSSMAN, Y. L., HODSON, R. E., LENSKE, R. E., MACK, R. N., AND REGAL, P. J. (1989). The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. *Ecology* **70**, 289–315.

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