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Biosafety Assessment of Microbial Strains Used in Biotechnology According to Their Taxonomy

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Abstract

A great variety of biotechnological products are now widely used in different ways in agriculture, medicine, food manufacturing and other areas of our life. Industrialized societies now more than ever depend on the use of genetically engineered products, with many of them synthesized using recombinant strains of microorganisms. There is an opinion that microbial strains used in biotechnology are potentially harmful for human health and the environment. Similar to many other countries, we have enacted environmental legislation in an effort to balance the risks and benefits of using biotechnological strains. Although environmental monitoring rules focus mainly on safety assessments of chemicals, the biosafety assessment of microbial strains used in biotechnology is a very important issue as well.

This article summarizes 15 years of research on the biotechnological strains of microbes widely used as producers of various biological substances for industrial purposes, and their environmental and biotechnological applications. In our survey, we tried to evaluate possible adverse effects (general toxicity and damage to the immune system, potential sensitizing effects, and damage to normal microbiota) caused by these microbes. It was shown that microscopical fungi of genera *Aspergillus*, *Penicillium* and *Candida*, and some gram-negative bacteria can affect the immune system and disrupt the normal balance of microbial flora of the intestinal tract in rats. The actinomycetes are less dangerous in that they cause fewer side effects. The investigation data obtained can be used to develop safety and hygienic standards for industrial microbes that will help decrease or minimize the occupational risk of infection or damage to the immune system when working with biotechnological strains of microbes. **(Int J Biomed. 2017; 7(1):51-56.)**

Key words: biotechnology • microbial strains • biosafety standards • hygiene regulations

Introduction

Without any doubt, biotechnology and numerous biotechnological products are valuable for medicine, veterinary science, agriculture, the food industry and other spheres of our life. Industrialized societies now more than ever depend on the use of genetically engineered products. Achievements of modern genetic engineering and molecular biology promote expansion of a wide range of the microbial strains used in the industry.

However, there is a concern that microbial strains used in biotechnology are potentially hazardous for human health and can increase the risk of biological environmental pollution.[1-3] Numerous investigations in Russia testify to the extensive influence of biotechnological strains on human health represented by allergic diseases of the respiratory organs, skin, and other immune disorders; and disruption of the normal flora balance of the body common to people working in the microbiological industry and living in residential areas, and even to those people who simply use some products of biotechnology.

Determining the safety of biotechnological strains of microorganisms and assessing the potential risk of the emission of 'strain producers' and their effect on the population can help prevent the technogenic pollution of residential areas, and it is evidently an extremely important issue. Many countries have enacted environmental legislation in an effort to balance the risks and benefits of using chemicals and biotechnological strains. Many surveys in Russia have investigated potential adverse effects of industrial strains of microbes.[4-6] These

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studies help develop safety and hygienic standards.

The results of research into the nature of harmful effects of industrial microorganisms helped outline the main stages and the scheme for researching hygienic rationing of microorganisms. However, these studies were developed without considering biological features of different taxonomic groups of microorganisms. The principles of hygienic standardization require a comprehensive approach and improvement of methodology. Our 15-year research was aimed at studying possible adverse effects of different taxonomic groups of biotechnological strains of microbes experimentally.

Material and methods

We have tested 32 strains of microorganisms applied in biotechnology as producers of a variety of biological substances. They included members of different taxonomic groups: gram-positive and gram-negative bacteria, actinomycetes, molds, and yeasts (Table 1).

The experiments were carried out on conventional male and female white mice (20-25 g, body weight) and Wistar rats (290-320 g, body weight). Each test and control group included 8 animals. The Institutional Ethical Committee of Animal Care and Use of Pirogov Russian National Research Medical University approved all procedures involving animals. After finishing each experiment, the animals were euthanized to prevent unnecessary suffering according to recommendations of the Ethical Committee of Animal Care and Use of Pirogov Russian National Research Medical University using an overdose of sodium pentothal.

To determine the virulence of biotechnological strains, we injected each species (mouse/rat) intraperitoneally with a dose of microbes equal to DV_{50} and examined the ability of the microbes to reach the bloodstream within 30 min after inoculation. The possible risk of microbes causing a generalized effect was evaluated by inoculation of culture media with 'fingerprints' of kidney, liver and spleen of animals on the 3rd, 8th, 15th and 22nd day after injection of the strain tested. [6,7]

Table 1.

Taxonomy of biotechnological strains of microorganisms used in Russia for industrial purposes and their characteristics

Strain	Activity/application
1. **Gram-positive bacteria:** *Bacillus subtilis* 65, *B.subtilis* 72, *B.subtilis* 103, *B. subtilis subsp. subtilis* KO-1 BKM B-2716 D, *B.licheniformis* 60, *B.licheniformis* 103, *B.licheniformis* B-9608, *B.licheniformis* KO-2 BKM B-2717D, *B.amiloliquefaciens* B-1029	Produce full-range complex of heat-stable amylolytic and proteolytic enzymes
Bacillus licheniformis 1001	Bacitracin-producing bacterium
Bacillus thuringiensis	Component of insecticide
Lactobacillus casei 5-1/8, *L. plantarum* 435, *Micrococcus varians* 80	Bacteria are used as 'fermenting agents' in meat product manufacturing
2. **Gram-negative bacteria:** *Alcaligenes denitrificans* C-32	Produces nitrilase
Pseudomonas caryophylii KM 92-102/1	Stirol destructor
3. **Actinomycetes** *Rhodococcus corallinus*	Purification of tobacco wastes
Rhodococcus erythropolis КД	Purification of environment from oil pollution
Streptomyces aureofaciens 777	Produces Biovit and Chlortetracycline for veterinary use
Streptomyces avermitilis 3NN	Produces Avermectin, the broad-spectrum antiparasitic agent
Streptomyces fradiae БС-1	Produces tylosin used in veterinary medicine
4. **Micromycetes and Yeasts** *Candida tropicalis* Y-456	Xylitol producer
Yarrowia lipolytica Y3323	Produces lipase
Tolypocladium cylindrosporum	Component of insecticide
Aspergillus terreus 44-62	Produces lovastatin
A.awamori 120/177, *A.awamori* Nakazawa ВУД Т-2 1000-У	Produce glucoamylase
P. funiculosum 18.2, *P.funiculosum* BKM F-3668D, *P.verruculosum* PV2007 BKM F-3972D	Produce a group of carbohydrases
Penicillium funiculosum F-149	Produces dextranase
Penicillium canescens PhPl33 BKM F3867	Produces pectin lyase (pectolyase) used in food industries and phytase used as an animal feed supplement
Trichoderma viride 44-11-62/3, *T. longibrachiatum* TW-1, *T. longibrachiatum* TW-420 BKM F-3880D	Producers of a range of cellulolytic enzymes, β-glucanase and xylanase
Trichoderma reesei 18.2K	Produces Celloviridin G20X used as an animal feed supplement

The general toxicity of each microbial strain in intranasal inhalation of animals within one month (with concentration of microbial suspension from 10^3 to 10^8 CFU/m^3) was determined by monitoring the body weight gained or lost, behavioral reactions of the animals, and total weight of internal organs upon termination of the experiment. To determine the dose of inoculation we used the following formula [8]:

$$C = \frac{D}{m \times t \times v \times 10^6}$$

C – the number of microbial cells per 1 m^3 of ambient air measured in CFU/m^3;

D - the number of microbial cells inoculated to animal measured in CFU per animal;

m – weight of the animal, g;

t – time of exposure, in minutes (it is equal to 240 and 120 min per day for rats and mice, respectively);

v – the pulmonary ventilation rate expressed as cm^3/(g x min) (it is taken as 0.65 and 1.24 for rats and mice, respectively);

10^6 – cm^3 to m^3 conversion coefficient.

The experiments concerning possible adverse effects to the immune system included measuring the total weight of immunocompetent organs, calculating leukocytes in Giemsa-stained blood smears, and also detecting and counting the main populations of T- and B-lymphocytes.[6-8]

The sensitizing effect on the development of delayed Type IV hypersensitivity reaction was demonstrated by means of inoculation of 10ml of sonicated microbes in a concentration of 10^4 CFU/ml in Freund's adjuvant (1:1). The inoculation was given under the aponeurosis of the animals' back feet with subsequent measurement of thickness of both back feet of each animal to detect possible edema due to allergic reaction. The immediate type of hypersensitivity reaction was shown by the effect of degranulation of mast cells taken from peritoneal exudates of rats. [7,9]

We conducted a bacteriological examination of feces to demonstrate possible adverse effects to gut microflora by inoculating 10-fold dilutions of animal feces in sterile saline onto a set of general-purpose and selective culture media for Enterobacteriaceae members, staphylococci, enterococci, clostridia, bifidobacteria, lactobacilli, and fungi, with subsequent identification of the genus of the microbes. [10,11] The specimens were taken immediately after the animals received microbial strain inoculation and 2 weeks thereafter.

The results of experiments were analyzed with simple t-test using Statistica (v.6.0, Stat Soft, USA) and Microsoft Office Excel 2007. Results were considered statistically significant when $P<0.05$. [12]

Results and Discussion

Our experiments have shown that the biotechnological strains we tested were not virulent, toxigenic or dangerous for warm-blooded animals. The virulence of microbes examined is also very low and LD_{50} is higher than 10^{12} CFU/animal (Table 2).

Table 2.

Virulence of biotechnological strains of microorganisms in intraperitoneal injections to rats

Microorganisms	LD50, lg CFU/ml	Threshold dose (limit dose), lg CFU/ml	Isolation from internal organs	
			Dose, lg CFU/ml	TAI, days
Gram-positive bacteria *(Bacillus spp.), Actinomycetes (Rhodococcus spp., Streptomyces spp.)*	>12.0	10.0 - 11.0	9.0 - 12.0	2
Gram-negative bacteria *(Alcaligenes sp., Pseudomonas sp., E. coli)*	>12.0	8.0 - 9.0	8.0 - 12.0	5
Micromycetes and Yeasts *(Aspergillus spp. Penicillium spp., Tolypocladium sp., Candida sp.)*	>12.0	9.0 - 10.0	9.0 - 12.0	8

TAI- time after inoculation

The invasiveness of gram-positive bacteria, actinomycetes, and spores of microscopic fungi is low; they do not multiply in internal organs of rodents and cannot persist in the host organism. The limit dose of these taxonomic groups of microbes that induced their translocation into blood at intraperitoneal injection was also very high and amounted to 10^9 CFU of fungal spores per animal, 10^{10} CFU/animal for actinomycetes, and 10^{11} CFU/animal for gram-positive bacteria.

As for gram-negative bacteria, their invasiveness seems to be relatively higher and the dose of microbes that caused bacteriaemia was about 10^8-10^9 CFU/animal (Table 2). We isolated colonies of gram-positive bacteria and actinomycetes from internal organs (kidneys, liver and spleen) within 2 days after inoculation, unlike gram-negative bacteria and fungi that continue to persist for up to 8 days. This fact testifies to the low invasive properties of these microbes.

In 'subchronic experiments' there was no visible toxic effect if the concentrations of microbes were inoculated for one month. Long-term inhalation of microorganisms tested may result in a mild immonotropic effect or light changes in the composition and concentration of normal intestinal flora habitants. Such effect was caused by the taxonomic group of microbes.[13-39]

In the case of a hygienic regulation of industrial microorganisms, it is required to define a possible sensitizing effect as the limiting criterion of harmful action. Our experiments have shown that 75% of examined bacteria, 67% of actinomycetes, and 80% of fungi (micromycetes) possess a sensitizing effect. Molds and yeasts, for example, demonstrated an immediate type of hypersensitivity that was dose-dependent. *Penicillium funiculosum* and *Aspergillus awamori* in concentrations of $2x10^4$ and $2x10^5$ CFU/m^3, respectively, caused intense degranulation of mast cells (Figure 1). *Bacillus licheniformis* caused a similar effect only if they reached the concentration of $5x10^6$ CFU/m^3 (Table 3).

The delayed Type IV hypersensitivity was typical for *Bacillus licheniformis*, *Pseudomonas caryophylii, Alcaligenes*

denitrificans and micromycetes in concentrations similar to immediate reactions. Actinomycetes did not show any sensitizing effect.

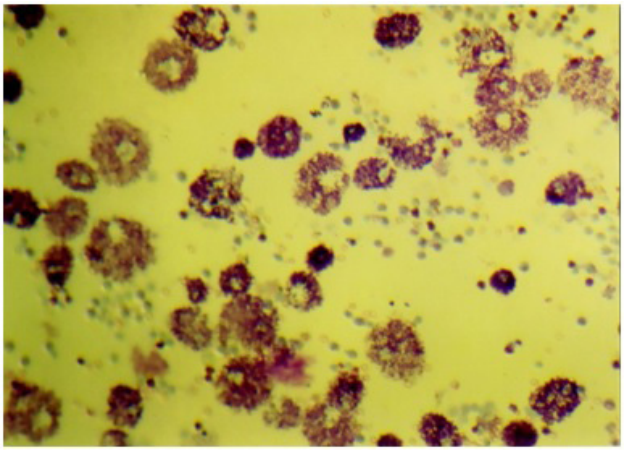

Fig. 1. *Degranulation of peritoneal mast cells in the case of A.awamori inoculation (2x10⁴ cells/m³), Giemsa Stain, x 400*

Furthermore, fungi and gram-negative bacteria affected the immune system, which resulted in decreasing the total amount of T-lymphocytes in peripheral blood and simultaneously increasing the number of B-cells (Table 3).

The microecological changes in the fecal microflora also varied greatly from strain to strain tested and was related to the taxonomic group of microorganisms (Table 4).

Table 4.

Characteristics of possible microecological disorders of large intestine caused by microbial strains used in biotechnology (ways of inoculation: oral and/or inhaling)

Microorganisms	Route of inoculation and dose of inoculation, lg CFU/m³	Microecological Changes
Gram-positive bacteria	5.0 – 7.0	Mild
Gram-negative bacteria	4.0	Manifest, severe
Actinomycetes	4.0 – 7.0	None
Candida sp.	3.0 – 4.0	Manifest, severe
Yarrowia sp.	3.0	None
Micromycetes (Aspergillus sp., Penicillium sp.)	4.0	Manifest, severe or intermediate
Micromycetes (Trichoderma sp.)	5.0	None

The most prominent visible effect was caused by *Candida sp.* and gram-negative bacteria in moderate inoculated concentrations – 10^3-10^4 CFU/m³.

Table 3.

Immunotropic activity of microbial strains used in biotechnology

Concentration of microorganisms, CFU/m³	T-lymphocytes, %	B-lymphocytes, %	Sensitizing effect		Eosinophils of Peripheral Blood, %
			Immediate type, %	Delayed type, mm	
R.erythropolis КД					
Control	44.2±1.5	17.8±1.5	4.7±1.2	0.142±0.007	2.5±0.6
Test 5x10⁷	39.6±2.3	20.2±1.4	5.7±1.0	0.151±0.010	2.6±0.4
B.subtilis 103					
Control	46.5±0.9	16.8±1.0	3.6±0.6	0.13±0.013	1.7±0.2
Test 1 - 6x10⁴	44.2±1.4	17.2±1.2	4.6±1.7	0.20±0.007	2.0±0.4
Test 2 - 6x10⁵	42.8±1.7	17.6±1.5	9.1±2.8	0.29±0.022	2.3±0.5
B.licheniformis 60					
Control	44.0±1.1	21.8±0.9	4.6±0.4	0.12±0.03	2.6±0.5
Test 1 - 5x10⁴	45.5±1.5	20.4±1.0	4.4±0.5	0.22±0.03	4.6±0.7
Test 2 - 5x10⁵	39.1±1.0*	25.4±1.7	9.0±0.9**	0.38±0.02**	5.5±1.1*
P. caryophylii KM 92-102/1					
Control	43.9±2.0	18.4±2.3	4.0±0.9	0.139±0.021	3.4±0.8
Test 1 - 7x10³	40.5±2.1	18.9±2.1	6.1±1.6	0.233±0.064	4.8±1.5
Test 2 - 7x10⁴	33.2±1.9**	22.5±1.7*	12.6±2.4**	0.403±0.034*	6.4±1.0*
P. canescens PhPl33 BKM F3867					
Control	41.6±0.8	21.1±0.8	3.5±0.6	0.19±0.03	3.4±0.3
Test 1 - 2x10³	39.8±0.4	22.2±1.8	4.2±0.5	0.24±0.04	4.2±0.8
Test 2 - 2x10⁴	36.3±1.2*	27.0±1.4*	12.5±2.7**	0.36±0.04*	7.2±1.2**
A. awamori 120/177					
Control	43.5±2.3	21.0±1.1	4.4±0.4	0.188±0.018	2.6±0.4
Test 1 - 1.2x10⁴	32.2±1.7**	24.6±0.9	11.0±1.1**	0.138±0.028	4.9±0.8*
Test 2 - 1.2x10⁵	29.6±3.5**	30.4±1.4**	24.4±2.7**	0.306±0.044*	4.4±0.5*
C.tropicalis Y-456					
Control	42.2±2.6	19.5±1.6	4.7±0.4	0.201±0.045	3.8±0.8
Test 1 - 3x10³	38.7±1.7	25.5±1.7*	11.2±1.8**	0.436±0.061*	8.3±1.1**
Test 2 - 3x10⁴	35.5±1.7	31.3±2.1**	20.0±3.3**	0.490±0.051*	10.0±1.5**

** P<0.05, ** P<0.01*

These changes observed in gut microbiota concerned the decreased level of normal lactose-positive *E.coli* and lactobacilli and simultaneously the increased concentration of opportunistic bacteria – staphylococci, enterococci, and gram-negative enterobacteria.

The mold strains of *A.awamori* and *P.funiculosum* at the level of $2x10^4$ CFU/m^3 also induced a decreased amount of normal fecal flora members and an elevated concentration of opportunistic enterobacteria, including *Proteus sp.* At the same time, the micromycetes of genus *Trichoderma* in higher inoculating concentrations did not demonstrate any visible side effects to gut microflora.

The gram-positive bacteria of genera *Lactobacillus, Micrococcus* and *Rhodococcus*, like *Bacillus sp,* and *Streptomyces sp.*, did not affect the immune system and gut microbiocenosis in very high concentrations (10^5 CFU/m^3) in inhaled air – 10^6, 10^7 and 10^8 CFU/m^3. The data obtained were used to improve the scheme of testing industrial strains and developing safety and hygiene standards for industrial use that will help decrease or minimize the occupational risk of infection and damage to the immune system when working with biotechnological strains of microbes. The strain producers of *Pseudomonas sp., Alcaligenes sp.,* fungi of genera *Aspergillus*, *Penicillium* and *Candida* make the list of priority industrial microorganisms for potential harm due to their sensitizing effect. We suggest testing any new biotechnological strain to determine its potential harmful effect according to standards developed. It is necessary to assess any possible adverse effects of newly prepared industrial strains of microorganisms.

The program of experiments can be shortened if performed for gram-positive candidates for industrial strains – including *Bacillus sp., Streptomyces sp.,* and *Trichoderma sp*. – that possess poorly expressed sensitizing properties. For the members of genera *Lactobacillus, Micrococcus, Rhodococcus,* etc., not showing harmful effects in the studied concentrations, we recommend performing group testing (for genus characteristics) on the basis of a study of their potential virulence.

Thus, our 15-year investigation allowed characterizing the degree of safety of microorganisms and creating a databank according to their toxicity and potential danger. The experimental justification for a maximum concentration limit of microorganism producers in the air of a working zone and atmospheric air of the occupied places was fixed in the State hygienic standards approved by the Federal Service for Supervision over Protection of Consumer Rights and Human Well-Being. [11, 32, 35-38]

Competing interests

The authors declare that they have no competing interests.

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