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EXPERIMENTAL ARTICLES

An Extracellular Protein of Propionic Acid Bacteria Inhibits Induced Mutations in *Salmonella typhimurium* Strains

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Abstract—A culture of propionic acid bacteria grown in a glucose-containing minimal medium, as well as the culture liquid and logarithmic-phase cells obtained from this culture, were found to inhibit the base pair substitution mutations induced by 4-nitroquinoline *N*-oxide, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, and sodium azide and the frameshift mutations induced by 9-aminoacridine. The antimutagenic activity of the culture liquid (CL) was presumably due to the presence of an extracellular thermolabile protein with a molecular mass of no more than 12 kDa, as evidenced by the facts that this activity considerably decreased after the treatment of the CL with pronase, its heating at 92°C, and its dialysis in a cellulose sack, which retains substances with molecular masses greater than 12 kDa. The residual antimutagenic activity of the dialyzed culture liquid was probably related to the interaction of the mutagen with thiols, rather than to the presence of organic acids (acetic or propionic). Thiols may also contribute to the antimutagenic activity of the *Propionibacterium shermanii* cells.

Key words: propionic acid bacteria, antimutagenesis, mutagens, extracellular protein, culture liquid.

Human activity gave rise to many artificial mutagens in addition to those which have been present in nature for a long time, and to which organisms managed to develop the respective protective mechanisms.

The natural antimutagens of humans and microbes play a decisive role in the protection of their DNA from damage and the preservation of their original genotype. The bacterium *Lactobacillus acidophilus* was found to reduce the activity of β-glucosidase, β-glucuronidase, nitrate reductase, and the reductases of azacompounds, which can transform some promutagens into mutagens and carcinogens [1]. Antimutagenic activity was reported for bifidobacteria [2] and propionic acid bacteria (PAB) [3]. It should be noted that lactic acid bacteria, bifidobacteria, and, potentially, propionic acid bacteria, are efficient human probiotics. PAB are tolerant to bile acids and low pH values and reduce the activity of the aforementioned bacterial enzymes involved in the formation of mutagenic compounds [4]. Propionic acid bacteria, which are able to acclimatize in the human intestines, aid in curing enteral dysbacterioses [5].

The aim of the present work was to study the ability of propionic acid bacteria to inhibit the action of various mutagens and to investigate the possible mechanisms of the antimutagenic activity of these bacteria.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The bacterium *Propionibacterium freudenreichii* subsp. *shermanii* VKM B-103 used in this work was maintained at the Department of Microbiology of the Faculty of Biology at Moscow State University. The bacterium was grown at 30°C in a medium containing (%) glucose, 1.5; $(NH_4)_2SO_4$, 0.3, KH_2PO_4, 0.1, Na_2HPO_4, 0.2, $CaCl_2$, 0.002, $MgSO_4$, 0.002; NaCl, 0.002; $CoCl_2$, 0.001; Difco tryptone, 0.1; and Difco yeast extract, 0.05. The medium was prepared using distilled water. The initial pH of the medium was 6.8–7.0. The organic acids formed during cultivation were regularly neutralized by adding the necessary amounts of 10% NaOH.

Exponential-phase cells were harvested by centrifugation at 10000*g* for 20 min, washed twice with 0.05 M Na–phosphate buffer (pH 7.0), and resuspended in the same buffer. This cell suspension was used as a genetic modulator (antimutagen) under the designation "cells." The liquid, referred to as culture liquid (CL), that remained after cell removal was also tested for antimutagenic activity. The whole culture of propionic acid bacteria was the third modulator type. In some experiments, the dialyzed fraction of the cytoplasmic proteins of *P. shermanii* cells, which was prepared as described earlier [3], was also tested for antimutagenic activity. Antimutagenic activity was assayed using the *Salmonella typhimurium* TA100 and TA97 cultures obtained

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Table 1. Antimutagenic activity of the culture liquid, cells, and dialyzed cell extract of *P. shermanii* with respect to the NQO-induced mutagenesis of *S. typhimurium* TA100

Preparation	Incubation time, h	The number of revertants per dish (X ± *SE*)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	24	86 ± 2.0	
Mutagen + Buffer	24	1059 ± 16.2	0
Sterile growth medium	24	889 ± 5.8	17.5
Culture liquid	24	118 ± 2.4	96.7
Cells	24	90 ± 4.0	99.0
Dialyzed cell extract:			
20 μg protein per dish	24	861 ± 6.6	20.3
20 μg protein per dish	48	884 ± 6.9	17.9
3 μg protein per dish	48	896 ± 6.1	16.8
0.5 μg protein per dish	48	847 ± 3.5	21.8

Note: Optical density (OD_{540}) of the *S. typhimurium* cell suspension was 0.5. In all experiments, the amount of culture liquid was 0.1 ml per dish.

from B.N. Ames (University of California, United States).

Reverse mutations were studied using Vogel–Bonner glucose agar (VBG agar).

Protein concentration was quantified by the method of Lowry *et al.* [6].

Mutagens used in this work were 4-nitroquinoline *N*-oxide (NQO), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), sodium azide (NaN_3), and the intercalate 9-aminoacridine (9-AA). All of these compounds were purchased from Sigma. NQO, MNNG, NaN_3, and 9-AA were used in amounts of 0.125, 1, 3, and 50 μg per petri dish, respectively. Stock solutions of NQO and 9-AA were prepared by dissolution in dimethyl sulfoxide (DMSO) at concentrations of 1 and 500 μg/ml, respectively. Immediately before experiments, the stock solutions were diluted to the required concentrations with 0.05 M phosphate buffer (pH 7.4). Solutions of MNNG and NaN_3 were prepared by dissolving them in 0.05 M citrate buffer (pH 6.2) and 0.05 M phosphate buffer (pH 7.4), respectively.

Antimutagenic activity was assayed by a modified method of Maron and Ames [7] as described earlier [3]. This method is based on the ability of mutagens to induce histidine revertants that are capable of growing on the respective selective medium, whereas antimutagenic modulators reduce the number of the revertants. Before introducing a modulator (the culture liquid, *P. shermanii* cells, etc.) into the test medium, it was incubated at 37°C for 20 min with a certain concentration of the mutagen (the exception was incubation with NQO, which lasted 24 or 48 h). Then, the mixture of the mutagen and modulator was placed with the test culture *S. typhimurium* in a tube with molten soft agar. The contents of the tube were mixed and poured into petri dishes containing layers of hardened, bottom agar medium [7]. After 48 h of incubation, *S. typhimurium* began to produce colonies of histidine revertants on the agar surface, while *P. shermanii* colonies failed to grow on this medium, because of the absence of thiamine and pantothenic acid two vitamins essential to the growth of propionic acid, bacteria. The positive control medium contained the same volume of the buffer instead of the modulator. The antimutagenic effect of the modulator (i.e., its mutation-inhibiting activity) was calculated by the following formula:

$$\text{Inhibition (\%)} = \frac{(a-b)\times 100}{a-c},$$

where *a* is the number of the histidine revertants induced by a mutagen, *b* is the number of the histidine revertants induced by the mutagen in the presence of an antimutagenic modulator, and *c* is the number of the histidine revertants grown in the presence of the modulator alone. In calculations, a correction for the number of spontaneous histidine revertants was made. All the mutagens studied were used in the concentrations that exerted no noticeable effect on the survival of the *S. typhimurium* test culture.

Data presented in the form $X \pm SE$ are the average of triplicate experiments. Standard deviation from the mean did not exceed 10%.

RESULTS

Inhibitory effect of *P. shermanii* preparations on NQO-induced mutagenesis. As can be seen from the data presented in Table 1, both the culture liquid and the cells of *P. shermanii* almost completely inhibited mutagenesis induced by NQO. The dialyzed cytoplasmic proteins of *P. shermanii* cells taken at concentrations from 0.5 to 20 μg per petri dish and incubated with NQO for 24 or 48 h did not exert any noticeable mutation-inhibiting effect. Nor did the sterile growth medium of *P. shermanii.*

It should be noted that the antimutagenic effects of the culture liquids prepared from 24- and 48-h *P. shermanii* cultures virtually did not differ, but the antimutagenic activity of the CL prepared from the older (72 h of growth) culture drastically decreased (data not shown). These data can be interpreted as indicating the utilization of antimutagen(s) in the late stages of bacterial growth.

The data presented in Table 2 give us an idea of some properties of the antimutagen. It may represent a thermolabile protein with a molecular mass of less than 12 kDa, as evidenced by the fact that the antimutagenic activity of the culture liquid, initially comprising 95.3%, decreased after its treatment with pronase, after heating at 92°C for 10 min, and after dialysis in a cellulose sack, which retains substances with molecular masses greater than 12 kDa, to 15.5, 25.2, and 4.5%, respectively.

Inhibitory effect of *P. shermanii* preparations on MNNG-induced mutagenesis. The culture liquid and cells of 24- and 48-h *P. shermanii* cultures diminished the mutagenicity of MNNG by about 55% (Table 3); i.e., the antimutagenic effect in this case was less pronounced than in the case of NQO. These results may indicate that the mechanisms of the interaction of the antimutagen with the NQO- and MNNG-induced test cells are different. The heating and dialysis of the culture liquid greatly diminished its antimutagenic activity, and treatment with pronase decreased it by about 42% (Table 4).

Inhibitory effect of *P. shermanii* preparations on NaN_3-induced mutagenesis. As can be seen from the data presented in Table 5, the culture liquid, culture, and cells of *P. shermanii* considerably decreased the number of the spontaneous and NaN_3-induced revertants of *S. typhimurium* TA100. The culture liquid alone (without the mutagen) virtually did not affect the number of the spontaneous revertants. Therefore, the culture liquid of *P. shermanii* does not exert any effect on the growth of the test *S. typhimurium* culture.

Treatment with pronase decreased the antimutagenic activity of the culture liquid from 92.8 to 10.1% (Table 6), providing further evidence for the protein nature of the *P. shermanii* antimutagen(s). On the other hand, heating at 92°C and dialysis decreased the antimutagenic activity of the culture liquid by about 50 and 75%, respectively. This suggests that, in addition to a dialyzable protein, the culture liquid of *P. shermanii* contains one additional compound of protein or nonprotein nature with antimutagenic activity. This suggestion is confirmed by the results of experiments with NQO, which indicate that this protease is unable to completely inactivate the antimutagenic activity of the culture liquid (Table 2).

Inhibitory effect of *P. shermanii* preparations on 9-AA-induced mutagenesis. Unlike the mutagens considered above, 9-AA induces frameshift mutations. As can be seen from Table 7, the culture liquid of

Table 2. Effect of heating (92°C, 10 min), dialysis, and proteolysis on the antimutagenic activity of the culture liquid of *P. shermanii* with respect to the NQO-induced mutagenesis of *S. typhimurium* TA100

Preparation	The number of revertants per dish ($X \pm SE$)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	146 ± 4.9	
Mutagen + Buffer	1447 ± 35.0	0
Culture liquid:		
Untreated	208 ± 6.1	95.3
After heating	1141 ± 74.6	25.2
After dialysis	1416 ± 22.3	4.5
After treatment with pronase	1271 ± 4.8	15.5

Note: Propionic acid bacteria were grown for 48 h. The time of incubation with NQO was 24 h.

Table 3. Antimutagenic activity of the culture liquid and cells of *P. shermanii* with respect to the MNNG-induced mutagenesis of *S. typhimurium* TA100

Preparation	The number of revertants per dish ($X \pm SE$)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	104 ± 2.4	
Mutagen + Buffer	1885 ± 23.6	0
24-h culture liquid	898 ± 20.0	55.4
24-h cells	869 ± 16.8	57.0
48-h culture liquid	897 ± 31.6	55.4
48-h cells	554 ± 13.8	74.7

Note: The time of incubation with MNNG was 20 min.

Table 4. Effect of heating (92°C, 10 min), dialysis, and proteolysis on the antimutagenic activity of the culture liquid of *P. shermanii* with respect to the MNNG-induced mutagenesis of *S. typhimurium* TA100

Preparation	The number of revertants per dish ($X \pm SE$)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	102 ± 6.6	
Mutagen + Buffer	1526 ± 14.7	0
Sterile growth medium	1540 ± 23.3	0
Culture liquid:		
Untreated	802 ± 11.6	50.8
After heating	1516 ± 10.4	0.5
After dialysis	1513 ± 17.5	0.6
After treatment with pronase	1225 ± 51.2	21.5

Note: The time of incubation with MNNG was 20 min.

Table 5. Antimutagenic activity of the culture liquid, cells, and whole culture of *P. shermanii* with respect to the NaN_3-induced mutagenesis of *S. typhimurium* TA100

Preparation	The number of revertants per dish ($X \pm SE$)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	92 ± 1.5	
Mutagen + Buffer	368 ± 13.9	0
Sterile growth medium	408 ± 13.1	0
Culture liquid	87 ± 4.6	96.2
Cells	56 ± 2.9	106.8
Whole culture	51 ± 3.2	108.6
Buffer + culture liquid (without NaN_3)	76 ± 2.9	

Note: Propionic acid bacteria were grown for 48 h. The time of incubation with sodium azide was 30 min.

Table 6. Effect of heating (92°C, 10 min), dialysis, and proteolysis on the antimutagenic activity of the culture liquid of *P. shermanii* with respect to the NaN_3-induced mutagenesis of *S. typhimurium* TA100

Preparation	The number of revertants per dish ($X \pm SE$)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	92 ± 10.7	
Mutagen + Buffer	470 ± 8.3	0
Culture liquid:		
Untreated	119 ± 3.5	92.8
After heating	292 ± 13.2	47.1
After dialysis	400 ± 13.6	18.5
After treatmentwith pronase	426 ± 9.0	10.1

Note: For experimental conditions, see the notes to Table 5.

Table 7. Effect of heating (92°C, 10 min), dialysis, and proteolysis on the antimutagenic activity of the culture liquid of *P. shermanii* with respect to the 9-aminoacridine-induced mutagenesis of *S. typhimurium* TA100

Preparation	The number of revertants per dish ($X \pm SE$)	Antimutagenic activity, %
Control (the number of spontaneous revertants)	65 ± 2.4	
Mutagen + Buffer	491 ± 6.1	0
Culture liquid + buffer	77 ± 4.3	–
Culture liquid:		
Untreated	205 ± 7.8	67.1
After treatment with pronase	436 ± 5.3	12.9
After dialysis	346 ± 4.4	34.0
After heating	371 ± 7.4	28.1

Note: An overnight test culture was diluted 20-fold and incubated for next 4 h to increase its mutability. The time of incubation with 9-AA was 30 min.

P. shermanii diminished 9-AA-induced mutagenesis in *S. typhimurium* TA97 by 67.1%. Treatment with pronase, dialysis, and heating at 92°C for 10 min decreased the antimutagenic activity of the culture liquid to 12.9, 34, and 28.1%, respectively.

Evaluation of the role of organic acids as possible antimutagens. Lankaputhra and Shah [8] found that lactic acid bacteria and bifidobacteria, as well as some organic acids (butyric, acetic, lactic, and pyruvic) at a concentration of 1%, suppress the mutagenicity of some mutagens, including NQO and MNNG. In our experiments, acetic and propionic acids taken at a concentration of 1% inhibited NQO-induced mutagenesis by 24.9 and 18.6%, respectively, and MNNG-induced mutagenesis by 23 and 15.5%. The same organic acids extracted from the culture liquid of propionic acid bacteria and taken at the same concentration (1%) did not exert any noticeable antimutagenic effect (data not shown).

DISCUSSION

The antimutagenic activity of propionic acid bacteria, localized in the fraction of soluble cytoplasmic proteins, was first revealed in 1991 [3]. In the present work, we studied the antimutagenic activity of the whole culture of propionic acid bacteria, as well as the culture liquid, cells, and dialyzed cell extract, against the pair substitution mutations induced by NQO, MNNG, and sodium azide, and against the frameshift mutations induced by 9-aminoacridine. It should be noted that, unlike NaN_3 and 9-AA, NQO and MNNG are promutagens, which become active only when metabolically activated.

Experiments showed that the antimutagenic activity of the whole culture and culture liquid of propionic acid bacteria was mainly due to a thermolabile protein with a molecular mass of no more than 12 kDa. This inference was based on the following results. Pronase greatly diminished the antimutagenicity against all of the mutagens studied. The incubation of the culture liquid in a dialysis sack, which is known to retain substances with molecular masses greater than 12 kDa, led to the almost complete loss of its antimutagenic activity. Heating at 92°C for 10 min almost completely destroyed the antimutagenic activity of the whole culture and culture liquid of propionic acid bacteria with respect to MNNG and, to a considerable degree, with respect to the other mutagens under study.

The protein with antimutagenic activity was secreted by the exponential-phase *P. shermanii* cells cultivated in a minimal medium with glucose. The antimutagenic activity of the sterile growth medium was very low.

The mechanism of the antimutagenic action of the secretory protein of *P. shermanii* remains unknown. We can only suggest that it is a sensor protein generating a primary signal when in contact with electrophilic com-

pounds (the majority of mutagens are just such compounds) in cells. In the late stages of this process, the natural antimutagenic system of cells is activated, thus increasing their resistance to mutagens.

Another antimutagenic mechanism was described by Matar *et al.* [9], who found that the antimutagenic effect of *Lactobacillus helveticus* L819 with respect to NQO was due to the proteolytic activity of this species and the secretion of peptides into milk. The extracellular peptides of two *Streptomyces* strains were also found to suppress the mutagenicity of UV radiation and NQO [10]. The mechanism of the antimutagenic action of these peptides was not studied.

The extracellular dialyzable protein is not the only mutagenic factor of *P. shermanii*: the culture liquid of this bacterium subjected to dialysis or proteolysis retained some antimutagenic activity (at levels from 0.5 to 21%). The residual antimutagenicity of the culture liquid was probably due to the presence of nondialyzable proteins and nonprotein compounds, such as organic acids [8] and thiols [11]. It should, however, be noted that acetic and propionic acids extracted from the culture liquid of propionic acid bacteria possessed very low antimutagenic activity (less than 12%).

As noted above, many mutagens are electrophilic compounds; therefore, thiols can inhibit mutagens due to their nucleophilicity. Propionic acid bacteria excrete thiols in addition to sulfur-containing amino acids [12] and contain GSH transferase [13]. There is a correlation between the antimutagenic activity of PAB with respect to NQO and the cellular content of thiols [13], which possess antioxidant and nucleophilic properties and can activate various cytosolic enzymes, including those involved in DNA repair [14]. The presence of cobalt ions in the medium and in PAB cells can promote DNA repair [15]. All of these factors may be responsible for the antimutagenic activity of PAB cells. The possibility cannot also be excluded that the antimutagenic action of PAB is due to the adsorption of mutagens on the cell surface.

The study of the physicochemical properties of the antimutagenic protein of propionic acid bacteria and the mechanism of its interaction with cells is in progress in our laboratory.

REFERENCES

1. Rao, D.R., Pulusani, S.R., and Chawan, C.B., *Diet, Nutrition and Cancer: A Critical Evaluation. Vol. 11. Micronutrients, Nonnutritive Dietary Factors, and Cancer*, Reddy, B.S. and Cohen, L.A., Eds., Boca Raton: CRC, 1986, pp. 63–75.
2. Shenderov, B.A. and Manvelova, M.A., Microbiological Implications of Functional Nutrition, *Med. aspekty mikrobnoi ekologii* (Medical Implications of Microbial Ecology), Shenderov, B.A., Ed., Moscow, 1994, no. 7/8, pp. 10–19.
3. Vorobjeva, L.I., Cherdinceva, T.A., Abilev, S.K., and Vorobjeva, N.V., Antimutagenicity of Propionic Acid Bacteria, *Mutation Res.*, 1991, vol. 251, pp. 233–239.
4. Perez-Chaia, A., Zirate, G., and Oliver, G., The Probiotic Properties of Propionibacteria, *Lait*, 1999, vol. 79, pp. 175–185.
5. Sidorchuk, I.I. and Bondarenko, V.M., Selection of a Biologically Active Mutant of *Propionibacterium shermanii* and the Possibility of Its Use in Complex Therapy of Enteral Dysbacteriosis, *J. Hyg. Epidemiol. Microbiol. Immunol.*, 1984, vol. 28, pp. 331–338.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, no. 1, pp. 265–275.
7. Maron, D.H. and Ames, B.N., Revised Methods for the *Salmonella* Mutagenicity Test, *Handbook of Mutagenicity Test Procedures*, Kilbey, B.J. *et al.*, Eds., Amsterdam: Elsevier, 1984, 2nd ed., pp. 95–130.
8. Lankaputhra, W.E.V. and Shah, N.P., Antimutagenic Properties of Organic Acids from Probiotic Bacteria, *Mutat. Res.*, 1998, vol. 397, pp. 169–182.
9. Matar, C., Nadathur, S.S., Bakalinsky, A.T., and Goulet, J., Antimutagenic Effects of Milk Fermented by *Lactobacillus helveticus* L819 and Protease-Deficient Derivative, *J. Dairy Sci.*, 1997, vol. 80, pp. 1965–1970.
10. Osava, T.S., Namiki, M.S., Udake, S., and Kada, T., Chemical Studies of Antimutagens of Microbial Origin, *Antimutagenesis and Anticarcinogenesis: Mechanisms*, Shankel, D.M. *et al.*, Eds., New York: Plenum, 1986.
11. Ketterer, B., Protective Role of Glutathione and Glutathione Transferase in Mutagenesis, *Mutat. Res.*, 1988, vol. 202, pp. 343–361.
12. Vorob'eva, L.I., Stoyanova, L.G., and Alekseeva, M.A., Practical Use of Propionic Acid Bacteria, *Mikrobnye metabolity* (Microbial Metabolites), Zvyagintsev, D.G., Ed., Moscow: Mosk. Gos. Univ., 1979, pp. 88–100.
13. Vorobjeva, L.I., Khodjaev, E.Yu., and Cherdinceva, T.A., The Study of Induced Antimutagenesis of Propionic Acid Bacteria, *J. Microbiol. Methods*, 1996, vol. 24, pp. 249–258.
14. De Flora, S. and Ramel, C., Mechanisms of Inhibitors of Mutagenesis and Carcinogenesis: Classification and Overview, *Mutat. Res.*, 1988, vol. 202, pp. 285–306.
15. Kuroda, Y. and Inoue, T., Antimutagenesis by Factors Affecting DNA Repair in Bacteria, *Mutat. Res.*, 1988, vol. 202, pp. 387–391.