Microbiology, Vol. 69, No. 5, 2000, pp. 565–570. Translated from Mikrobiologiya, Vol. 69, No. 5, 2000, pp. 674–680.
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EXPERIMENTAL ARTICLES

Isolation and Characterization of *Acinetobacter* sp. Mutants Defective in Exopolysaccharide Biosynthesis

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Received November 4, 1999; in final form, March 16, 2000

Abstract—Nitrosoguanidine-induced mutants of *Acinetobacter* sp. defective in exopolysaccharide biosynthesis did not differ from the parent strain in distinguishing physiological and biochemical properties, such as requirements for growth factors, utilization of mono- and disaccharides, and resistance to antibiotics. The genetic relation of parent and mutant strains was shown by 16S rRNA PCR analysis. The comparative study of parent and mutant strains with respect to resistance to unfavorable environmental factors confirmed our hypothesis that *Acinetobacter* sp. exopolysaccharides perform protective functions. Hybridization experiments revealed the conjugal transfer of plasmid R68.45 from *Pseudomonas putida* BS228 (R68.45) to mutant but not to the parent *Acinetobacter* sp. strains. The role of the *Acinetobacter* sp. exopolysaccharides in providing the genetic stability of this bacterium is discussed.

Key words: *Acinetobacter*, exopolysaccharides, genetic stability

Acinetobacter sp. produces a high-viscosity exopolysaccharide (EPS) complex, called etapolan [1]. During the growth of the bacterium on a variety of substrates (ethanol, mono- and disaccharides, molasses, starch, C_4-dicarboxylic acids, etc), the viscosity of the culture liquid may reach 1000–1500 mPa s at a relatively low EPS content of 4–5 g/l. Etapolan cannot be separated from bacterial cells by mild treatment procedures, such as treatment with NaCl solutions, and this considerably impedes manipulations with *Acinetobacter* sp. In our earlier studies, *Acinetobacter* sp. cells were rid of etapolan with the aid of polysaccharide-degrading enzymes [2] or by ultrasonic treatment [3]. Alternatively, this can be achieved by obtaining mutants defective in exopolysaccharide biosynthesis.

The present work was aimed at the selection of such mutants (EPS^- mutants), investigation of the protective functions of etapolan, and the comparative genetic study of parent and mutant strains.

MATERIALS AND METHODS

Strains. *Acinetobacter* sp. strain 12S, resistant to 1 mg/ml streptomycin, was described earlier [1]. The plasmid-bearing strain *Pseudomonas putida* BS228 (R68.45) was a gift from A.M. Boronin (Laboratory of Plasmid Biology, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino). The pink-pigmented facultative methylotroph *Methylobacterium extorquens* 19ch was obtained from V.A. Romanovskaya (Collection of Microorganisms, Zabolotnyi Institute of Microbiology and Virology).

Cultivation conditions. *Acinetobacter* sp. strains were grown in liquid Kodama medium [4] supplemented with 1 vol % ethanol as the carbon and energy source, 0.5 vol % yeast extract, and 0.0003% calcium pantothenate (pH 6.8–7.0). Material for inoculum was 2-day culture grown on glucose–potato agar (GPA). Cultivation was performed at 30°C on a shaker (220 rpm) for 16 to 96 h. Alternatively, *Acinetobacter* sp. strains were grown on agar Kodama medium supplemented with 0.5 vol % ethanol and 0.2 vol % yeast extract.

M. extorquens 19ch was grown in liquid Kodama medium supplemented with 1 vol % methanol as the carbon and energy source. Material for inoculation was 2-day culture grown on agar Kodama medium with 0.5 vol % methanol. Cultivation was performed at 30°C on a shaker (220 rpm) to the exponential phase (16–20 h of growth).

P. putida BS228 (R68.45) was grown in nutrient broth to the mid-exponential growth phase (220 rpm; 30°C; 16–18 h). Material for inoculation was 1-day culture grown on nutrient agar.

Selection of EPS^- mutants. *Acinetobacter* sp. 12S cells grown to the exponential phase were harvested by centrifugation at 10000 *g* for 5 min and suspended in a 0.1-M citrate–phosphate buffer or mineral Kodama medium. Mutations were induced by treating cell suspensions with 1-methyl-3-nitro-1-nitrosoguanidine

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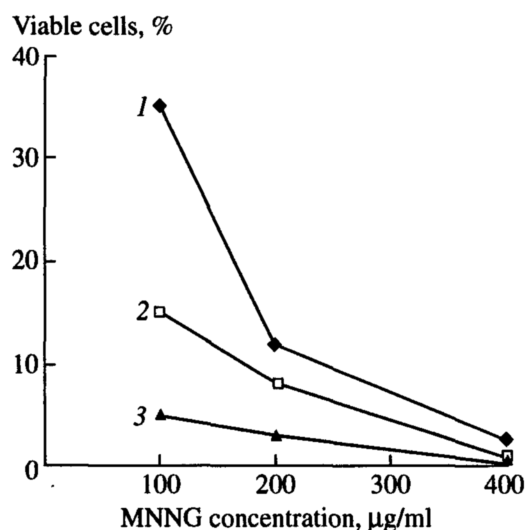

Fig. 1. Concentration dependence of the effect of nitrosoguanidine on the survival of *Acinetobacter* sp. 12S cells at different treatment times (min): (*1*) 15, (*2*) 30, and (*3*) 60.

(MNNG) as described in Miller's handbook [5]. Optimal conditions for mutagenesis were chosen by incubating a bacterial suspension containing 10^9 cells/ml in the presence of 25, 50, 75, 100, 200, and 400 µg/ml nitrosoguanidine for 15, 30, and 60 min at 30°C. After treatment, cells were washed free of MNNG with mineral Kodama medium or citrate–phosphate buffer by suspending and centrifuging them at 10000 *g* for 5 min and plated on agar Kodama medium with ethanol. EPS^- mutant colonies were selected according to their specific colonial morphotype: the mutant colonies were small, flat, and nonviscous. The clones chosen were transferred to ethanol-containing agar Kodama medium, wort agar, nutrient agar, and GPA in order to investigate their physiological and biochemical characteristics.

Physiological and biochemical characteristics of parent and mutant *Acinetobacter* sp. strains. To be sure that the clones chosen are *Acinetobacter* sp. 12S mutants and not contaminating microflora, they were analyzed for some typical properties, such as the requirement for growth factors, ability to utilize mono- and disaccharides, and antibiotic resistance. For this purpose, the mutant and parent strains were grown in liquid mineral Kodama medium with ethanol or one of the carbohydrates (glucose, fructose, mannose, rhamnose, maltose, and lactose) and containing or not containing growth factors (yeast extract and calcium pantothenate). The carbohydrates were added in amounts equivalent to that of ethanol with respect to carbon. The clones were grown to the stationary phase (96 h of growth) as described above.

Susceptibility to kanamycin, ampicillin, and chloramphenicol in an amount of 1 µg/ml and streptomycin in an amount of 1 mg/ml was tested by growing the clones on ethanol-containing agar Kodama medium in the presence of these antibiotics.

EPS in the culture liquid was detected by reaction with phenol and sulfuric acid [6] and by the precipitation of EPS from the culture liquid with ethanol or isopropanol.

16S rRNA PCR analysis of mutant and parent *Acinetobacter* sp. strains. DNA from mutant and parent cells was isolated by the method of Marmur [7]. The PCR-based amplifications of the 16S rRNA genes were carried out with the eubacterial forward primer 27f and reverse primer 1492r [8] in an MJ Research Inc. thermal cycler (United States). The reaction mixture (50 µl) contained 1 µl (100 ng) DNA, 5 µl buffer containing 100 mmol Tris–HCl, 500 mmol KCl, 25 mmol $MgCl_2$, and 0.01% gelatin (pH 8.3), 0.5 µl (100 mmol) deoxynucleoside triphosphates (25 mmol of each dNTP), 1 µl (50 nmol) of each primer, and 0.5 µl (1–2 U) Taq-polymerase (Gibco BRL). Each of the 30 PCR cycles was a standard three-step reaction, with DNA denaturation at 94°C for 60 s, primer annealing at 55°C for 60 s, and DNA synthesis at 72°C for 60 s.

The size of the amplicons synthesized by PCR was determined by subjecting PCR products to electrophoresis in 1% agarose gel with DNA molecular weight markers from Gibco BRL. PCR amplification products were then digested by the restriction endonucleases *Eco*RI and *Hae*III (Sigma), and the restriction fragments of PCR products were analyzed by electrophoresis under the same conditions as above.

Resistance of parent and mutant *Acinetobacter* sp. strains to stress factors was investigated as follows. Aliquots of cultures grown in liquid mineral Kodama medium to the exponential phase were ultrasonically treated at 22 kHz for 120 and 180 s or were incubated in the presence of 2.5 mM sodium dodecyl sulfate (SDS), 2 mM formaldehyde, 1.5 mM Cu^{2+}, or 7 mM Cr^{6+}, after which the number of viable cells in suspensions was determined on GPA by Koch's method. Formaldehyde and SDS were added to culture aliquots as 1 and 10% solutions, respectively. Cu^{2+} and Cr^{6+} ions were added in the form of 0.1 M solutions of $CuSO_4 \cdot 5H_2$ and $KCrO_4$.

In order to investigate the functional role of EPS, the resistance of parent cells to the above stress factors was also evaluated after EPS had been stripped off the cells by ultrasound as described earlier [3].

Conjugal transfer of plasmid R68.45 from *P. putida* BS228 to parent and mutant *Acinetobacter* sp. strains. The conjugal transfer of plasmid R68.45 (the P-1 incompatibility group) from *P. putida* BS228 (R68.45) to *Acinetobacter* sp. strains was investigated as follows. Cells of *Acinetobacter* sp. and *P. putida* BS228 (R68.45) grown on a shaker to the exponential phase were harvested by centrifugation at 10000 *g* for 5 min and suspended in Kodama medium without any carbon source (carbon-deficient medium). Hybridization

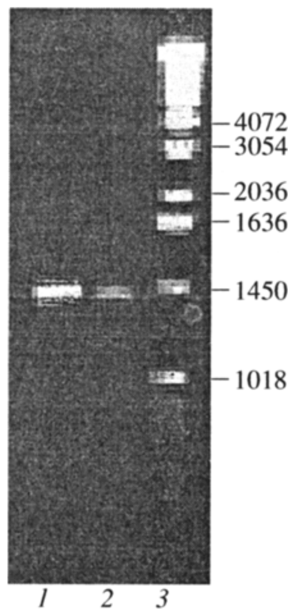

Fig. 2. Electrophoresis (1% agarose gel) of the PCR amplification products of (*1*) parent *Acinetobacter* sp. 12S and (*2*) EPS⁻ mutant 1. Lane *3* represents DNA molecular weight markers (numerals indicate the number of nucleotides).

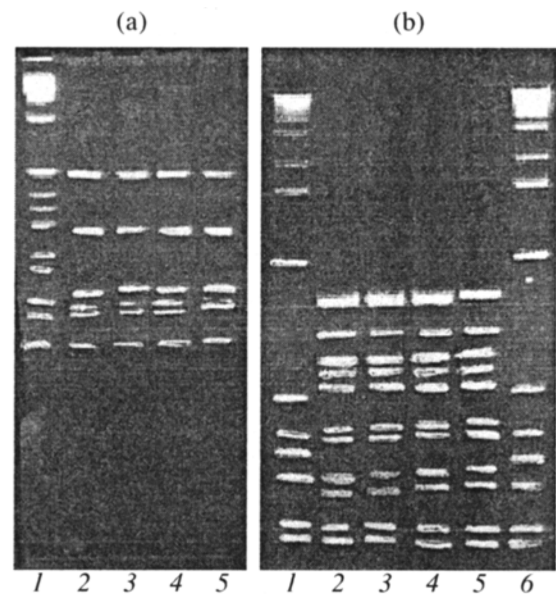

Fig. 3. Electrophoresis (1% agarose gel) of the PCR amplification products of (*2*) parent *Acinetobacter* sp. 12S and (*3–5*) EPS⁻ mutants 1, 5, and 6 digested with (a) *Hae*III and (b) *Hae*III and *Eco*RI mixture. Lanes *1*a, *1*b, and *6*b represent DNA molecular weight markers.

was performed either on carbon-deficient agar Kodama medium or in carbon-deficient liquid Kodama medium. In the former case, 0.1-ml aliquots of donor and recipient cultures, each containing 10^8–10^{10} cells/ml, were placed on the surface of carbon-deficient agar and incubated at 30°C for 16 h, after which cells were washed off of the agar with physiological saline, and the resultant suspension was plated, in 0.1-ml aliquots, on selective agar medium, whose composition is indicated below. In the latter case, donor and recipient cultures, each containing 10^9 cells/ml, were mixed in a proportion of 1 : 10 and incubated at 30°C without stirring for 16 h, after which the cell suspension was plated, in 0.1-ml aliquots, on selective agar medium. This medium contained ethanol as the carbon source, 1 mg/ml streptomycin (to prevent the growth of donor *P. putida* BS228 (R68.45) strain), and 20 µg/ml of each ampicillin and kanamycin (to prevent the growth of recipient *Acinetobacter* sp. strains, which were sensitive to ampicillin and kanamycin in an amount of 1 µg/ml). The transconjugants obtained were tested for susceptibility to 300 µg/ml ampicillin, 100 µg/ml kanamycin, and 40 µg/ml tetracycline.

The transconjugant frequency was calculated as the ratio of the number of transconjugant cells grown on selective medium to the number of viable *Acinetobacter* sp. cells in the cell suspension subjected to hybridization. Viable cell count was performed by Koch's method using agar Kodama medium with ethanol and 1 mg/ml streptomycin.

To be sure that the clones selected were in fact *Acinetobacter* sp. transconjugants, they were subjected to reverse hybridization with *M. extorquens* 19ch cells on carbon-deficient agar or in liquid Kodama medium, as described above. *M. extorquens* 19ch transconjugants were selected on agar Kodama medium containing 1 vol % methanol, 50 µg/ml ampicillin, and 50 µg/ml kanamycin, on which donor *Acinetobacter* sp. cells could not grow, because of their inability to utilize methanol, and recipient *M. extorquens* 19ch cells could not grow, because of their sensitivity to these antibiotics at concentrations of 10 µg/ml. The frequency of the conjugal transfer of plasmid R68.45 to *M. extorquens* 19ch cells was determined as described above, by counting viable methylotrophic cells on agar Kodama medium containing 1 vol % methanol.

RESULTS AND DISCUSSION

Experiments on the survival of *Acinetobacter* sp. 12S cells under the action of nitrosoguanidine showed that the percentage of viable cells in the suspension treated with 200 µg/ml MNNG decreased from 12 to 3%, as the treatment time was lengthened from 15 to 60 min (Fig. 1). Such survival rates are considered to be optimal for the mutagenesis induced by MNNG [5]; in view of this, EPS⁻ mutations were induced in *Acinetobacter* sp. cells by treating them with 200 µg/ml nitrosoguanidine. As a result, we selected ten clones producing small (1–2 mm in diameter), flat, dull, white, and nonviscous colonies, when grown on ethanol-containing agar Kodama medium, wort agar, GPA, and nutrient agar.

Like the parent *Acinetobacter* sp. strain, mutant clones could grow in liquid mineral medium with etha-

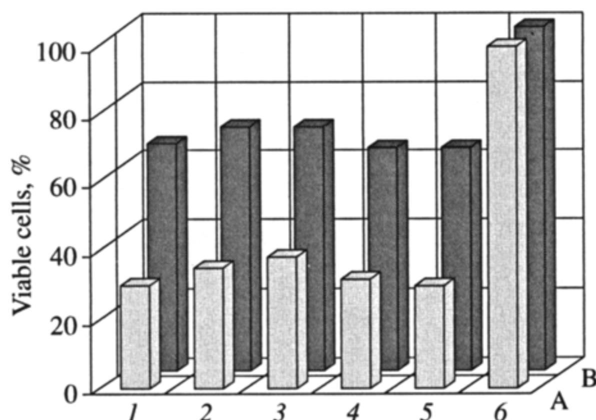

Fig. 4. Effect of ultrasonic treatment on the survival rate of (*6*) parent *Acinetobacter* sp. 12S and (*1–5*) EPS⁻ mutants 1, 3, 5, 6, and 9. Treatment time (s): (A) 180 and (B) 120.

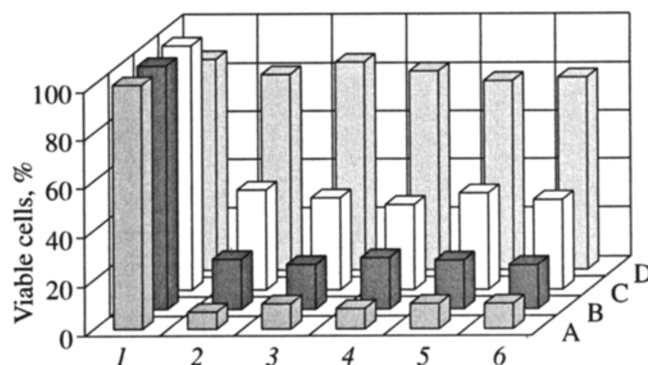

Fig. 5. Effect of (A) 2.5 mM SDS, (B) 2 mM formaldehyde, (C) 1.5 mM Cu^{2+}, and (D) 7 mM Cr^{6+} on the survival rate of (*1*) intact and (*2*) EPS-deprived parent *Acinetobacter* sp. 12S cells and (*3–6*) EPS⁻ mutants 1, 3, 5, and 9.

nol only if it was supplemented with calcium pantothenate and yeast extract. Both parent and mutant strains were able to utilize glucose, mannose, fructose, and lactose but failed to utilize rhamnose and maltose. The culture liquid of mutant strains grown on ethanol or hydrocarbons was nonviscous; in this case, the content of carbohydrates in the culture liquids of mutants was within 0.05–0.15 g/l, as compared with 2.0–2.5 g/l carbohydrates present in the culture liquid of parent *Acinetobacter* sp. Unlike the culture liquid of the parent strain, the culture liquid of mutants did not give rise to EPS precipitates when treated with organic solvents. Presumably, carbohydrates present in small amounts in the culture liquid of mutant strains represent extracellular mono- or oligosaccharides or, which is more likely, some cellular components of a carbohydrate nature released into the medium as a result of partial cell lysis in the stationary growth phase.

Mutant strains did not differ from the parent strain in antibiotic sensitivity: all strains turned out to be sensitive to kanamycin, ampicillin, and chloramphenicol at concentrations of 1 μg/ml and resistant to a high (1 mg/l) concentration of streptomycin.

Thus, the obtained results showed that mutant clones did not differ from the parent *Acinetobacter* sp. strain 12S in the requirements for growth factors, in the range of utilizable carbohydrates, and in antibiotic sensitivity, but did differ in their inability to synthesize EPS.

The genetic relationship between parent and mutant *Acinetobacter* sp. strains was investigated by analyzing their 16S rRNA genes by the multiplex randomly amplified polymorphic DNA PCR [9] with the eubacterial primers 27f and 1492r. Instead of sequencing the PCR amplification products, which is a common practice in the genetic identification of strains [10–12], we performed their restriction fragment length polymorphism (RFLP) analysis [13, 14].

Electrophoresis of the PCR products of the parent and mutant strains in 1% agarose gel (Fig. 2) showed that all the amplicons obtained had an identical size of 1450 nucleotides. In the subsequent RFLP experiments, the amplicons were digested with restriction endonucleases, and the digests were again analyzed by electrophoresis. In Fig. 3, panel (a) shows the results of electrophoresis of the PCR amplification products of the parent and three mutant (1, 5, and 6) strains digested with *Hae*III, and panel (b) shows the electrophoresis of the same PCR amplification products digested with a mixture of two endonucleases, *Hae*III and *Eco*RI. As evident from Fig. 3, the DNA fragment patterns of all strains analyzed were identical, indicating a genetic relation between the parent and mutant *Acinetobacter* sp. strains.

In experiments to study the susceptibility of the parent and mutant *Acinetobacter* sp. strains to stress factors, one of such factors was ultrasound. The data presented in Fig. 4 show that ultrasonic treatment for 120 and 180 s did not affect the viability of parent cells (Fig. 4, bars *6*) but decreased the number of viable cells in the cell suspensions of mutants 1, 3, 5, 6, and 9 by 40 to 80% (Fig. 4, bars *1–5*).

Unlike intact (i.e., not deprived of EPS) parent cells, which were resistant to all stress factors investigated (Fig. 5, bars *1*), EPS⁻ mutant cells and parent cells deprived of EPS by ultrasonic treatment showed low resistance to SDS, formaldehyde, and copper ions (Fig. 5, bars *2–6*). It should be noted that mutant and parent (both intact and EPS-deprived) cells exhibited similar resistance to Cr^{6+} ions; this suggests that *Acinetobacter* sp. possesses other mechanisms of resistance to heavy metal ions than those associated with EPS biosynthesis [15]. On the other hand, the data presented in Fig. 5 are further evidence of the protective effect of EPS on *Acinetobacter* sp. cells revealed in our earlier works [3, 15].

Along with exerting protection from stress factors, EPS might also reduce the probability of penetration of foreign plasmid and chromosomal DNA into *Acinetobacter* sp. cells, thus increasing their genetic stability.

Resistance of *Acinetobacter* sp. mutant 1, *P. putida* BS228 (R68.45), and their transconjugants to antibiotics

Strain	Noninhibitory concentrations, μg/ml		
	ampicillin	kanamycin	tetracycline
Acinetobacter sp. mutant 1 defective in EPS synthesis	0.5	0.5	0.5
P. putida BS228 (R68.45)	300	100	40
Acinetobacter sp. mutant 1 (R68.45) transconjugants	300	100	10

To check this assumption, we attempted to hybridize the parent *Acinetobacter* sp. strain 12S and its EPS^- mutants 1 and 3 with *P. putida* BS228 (R68.45), whose plasmid bears genes encoding resistance to ampicillin, kanamycin, and tetracycline at concentrations of 300, 100, and 40 μg/ml, respectively. Unlike the *P. putida* strain, *Acinetobacter* sp. 12S is sensitive to the above-mentioned antibiotics at a concentration as low as 0.5 μg/ml.

We failed to implement the conjugal transfer of plasmid R68.45 to parent *Acinetobacter* sp. 12S cells, whereas the hybridization of *P. putida* BS228 (R68.45) and EPS^- mutant cells gave rise to more than 50 *Acinetobacter* sp. transconjugants exhibiting resistance to all three marker antibiotics (see table). The frequency of plasmid R68.45 transfer to recipient *Acinetobacter* sp. mutant 1 and mutant 3 cells was less than 10^{-8} per recipient cell in liquid medium but reached 3×10^{-6} per recipient cell on agar medium.

In order to demonstrate the presence of plasmid R68.45 in the transconjugants of EPS^- mutants 1 and 3 of *Acinetobacter* sp., the transconjugants were subjected to reverse hybridization with the methylotroph *M. extorquens* 19ch, which is resistant to ampicillin and kanamycin at a concentration of 10 μg/ml and to tetracycline at a concentration of 5 μg/ml. Most of the 45 *M. extorquens* 19ch transconjugants obtained turned out to be resistant to ampicillin, kanamycin, and tetracycline at concentrations of 300, 100, and 25μg/ml, respectively. In this case, the frequency of plasmid R68.45 transfer was less than 10^{-7} per recipient cell in liquid medium and reached 2×10^{-5} per recipient cell on agar medium.

These data suggest the improved genetic stability of *Acinetobacter* sp. 12S due to the protective effect of EPS from the penetration of foreign DNA. This suggestion is supported by the fact that, over a period of 15 years of manipulations with this producer of etapolan under laboratory and pilot conditions, we did not observe any variability or phagolysis of this strain.

To the best of our knowledge, there is no published information concerning the effect of EPSs on the genetic stability of microbial populations, although there is evidence that the cell-associated polysaccharide emulsan is responsible for the resistance of *Acinetobacter calcoaceticus* RAG-1 strain to phages ap-2 and ap-3 [16, 17]. The mutants of this strain that acquired resistance to one phage retained their susceptibility to the other phage, the acquisition of resistance to phage ap-3 being accompanied by a suppression of emulsan production [16]. The mutants of RAG-1 defective in emulsan production turned out to be resistant to the phages [17]. These observations allowed the authors of these papers to suggest that emulsan is involved in phage adsorption and to explain the resistance of cells to phages by the inability of the latter to attach to the cell surface.

To conclude, we succeeded in obtaining *Acinetobacter* sp. mutants defective in EPS (etapolan) synthesis. The genetic relation between the mutant and parent *Acinetobacter* sp. strains was established based on the results of analysis of the main physiological and biochemical properties of this bacterium and 16S rRNA PCR data. The results obtained show that etapolan protects the producer from unfavorable environmental factors and may be responsible for its high genetic stability.

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