Microbiology, Vol. 74, No. 6, 2005, pp. 709–714. Translated from Mikrobiologiya, Vol. 74, No. 6, 2005, pp. 816–822.

EXPERIMENTAL ARTICLES

Genomic and Phenotypic Analyses of Microorganisms Isolated from the Sediments of Lake Baikal

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Received September 6, 2004

Abstract—Genetic and biochemical methods and morphological examination were used to study microorganisms isolated from samples of deep drilling of the Lake Baikal bottom sediments. Based on blot hybridization patterns, the strains investigated were divided into several groups according to the degree of homology of their genomic DNA. Morphological, biochemical, and ultrastructural characteristics of bacterial strains are described, and their compliance with the genomic analysis data is demonstrated.

Key words: microorganisms, identification, molecular systematics, G+C composition, genomic fingerprinting, ultrastructure of bacilli.

Identification of bacteria generally relies on morphophysiological, biochemical, and other phenotypic characteristics. Nevertheless, the methods of molecular systematics have been widely used over the last two decades for studying microorganisms. The G+C composition (mol %) of chromosomal DNA is among the main criteria in molecular systematics [1]. This trait, however, is often insufficient for species attribution of both known strains and those yet unidentified: several cases of coincidence of DNA G+C compositions for rather distant genera are reported. Genomic fingerprinting makes it possible to solve many important problems in determining the genetic diversity, identity, and relationship of various organisms at the level of DNA structure variation. In this analysis, molecular genetic markers are used as the main tools. Minisatellite DNA of various types is now considered to be the most highly efficient polymorphism markers [2, 3].

A nonradioactive variant of genomic fingerprinting, based on biotin-labeled single-strand M13 phage DNA [4, 5], has been developed for analyzing genomic polymorphism of bacterial strains.

The goal of this work was to study the correlations between genomic and phenotypic analyses of bacteria isolated from bottom sediments of Lake Baikal, with a view to determining their taxonomic attribution, and to evaluate the utility of various methods for identification of microorganisms.

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MATERIALS AND METHODS

Bottom sediments of Lake Baikal were sampled during drilling in the Shoal Posol'skaya; the coordinates are 52°05′27.1″ northern latitude and 105°50′23.5″ eastern longitude (1999). The maximum drilling depth was 320 m. The samples were taken aseptically from the central part of the core. The sedimentary deposits were mainly represented by clay, aleurite silts, diatomaceous silts, and diatomaceous shell sediments. As a result, a collection of microorganisms was assembled [6]. For this work, the strains from the collection were selected randomly.

Phenotypic traits of the strains and their resistance and/or sensitivity to antibiotics were determined conventionally [7, 8]. Specific features of the fine cell structure were examined by electron microscopy of ultrathin sections using an H-600 (Hitachi, Japan) electron microscope.

High-molecular-weight DNA was isolated as described earlier [5].

For hydrolyzing DNA, 5–10 μg DNA from each sample were digested with the corresponding restriction endonucleases (SibEnzym, Novosibirsk, Russia) for 17–18 h at a temperature of 37°C taking 1–2 AU of the enzyme per 1 μg DNA. DNA fragments were separated by electrophoresis, and the gels were blotted onto caprone filters [3, 4]. Upon Southern blotting onto the filters, the DNA fragments were hybridized with biotin-labeled M13 bacteriophage DNA [9].

PCR genotyping was performed as described in [10]. The reaction mixture (25 μl) contained 1.2 μl of the mixture of 2 mM nucleoside triphosphates, 2.5 μl of MPCR buffer (0.5 M Tris–HCl pH 8.8, 160 mM

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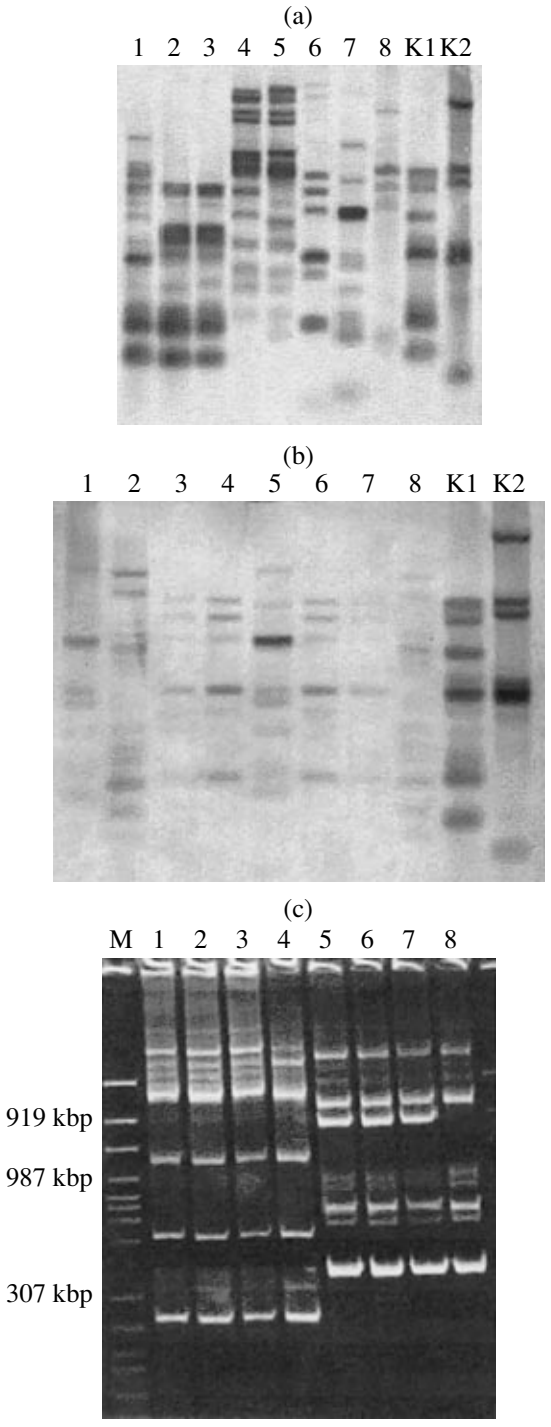

$(NH_4)_2SO_4$, 20 mM $MgCl_2$, and 0.5 mM Tween 20), 0.5 µl of *Taq*-polymerase, 2.5 µl of 0.02 M $MgCl_2$, 1 µl of oligonucleotide primer, and 1 ng of genomic DNA. The amplification conditions involved 30 cycles of 1 min at 94°C, 1 min at 42°C, and 1.5 min at 72°C and a final synthesis stage of 8 min at 72°C. The amp-licons were analyzed by PAGE (4%), followed by staining with ethidium bromide [10]. The genomic DNA of bacteria was amplified using two primers: 5'-AGGACGAGCTCGCGGATATA (N1) and 5'-GCAACATTTTGCTGCCGGTCAC (N3).

The nucleotide composition of DNA was determined by thermal denaturation in 0.1 × SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) in a spectrophotometer with a thermostated cuvette at a heating rate of 0.5°C/min. The G+C composition was calculated according to the following formula:

$$(\text{G+C})\ \text{mol}\ \% = 2.08 \times T_{\text{melt}} - 106.4,$$

where T_{melt} is the melting temperature of DNA sample assayed. The DNA of *Escherichia coli* B was used as a standard [7].

RESULTS AND DISCUSSION

Genomic polymorphism of the strains. Genomic fingerprinting produced strain-specific band patterns, which we used for comparison in an attempt to determine the degree of homology between bacterial DNAs and, which is most important, to ascertain the correlation between morphological, biochemical, and ultrastructural characteristics of these bacterial strains and their similarity at the genomic level. Based on blot hybridization patterns, all the strains studied were divided into 12 groups (table). Figure 1 shows genomic fingerprints of several Baikal strains listed in the table. When using biotinylated DNA of M13 phage, the number of minisatellite DNA fragments in the range of 1–5 bp varied from 6 to 15 per lane, on the average. For a more precise characterization of the strains, PCR genomic fingerprinting was additionally performed [10, 12]. The primers N1 and N3 were chosen for amplification of genomic DNA of bacilli, as they produced the most informative PCR fingerprinting patterns.

We succeeded in detecting a certain distinction for Che1008, Che1012, and Che1015, previously assigned to group I (Fig. 1c). Although this distinction is minor—an additional band with a length of 900 kbp in

Fig. 1. Genomic fingerprints of the Baikal strains. Panels (a) and (b): Genomic fingerprinting with biotinylated M13 phage DNA. Blot hybridization of the chromosome DNA of the microbial strains isolated from Lake Baikal after hydrolysis with *Eco*RI–*Hin*dIII restriction endonucleases. Panel (a): strains (*1*) LB-31, (*2*) LB-40, (*3*) LB-41, (*4*) LB-56, (*5*) LB-182: (*6*) Si84, (*7*) Si80, (*8*) LB-62, (C1) *B. subtilis* 652, and (C2) *E. coli* JM103. Panel (b): strains (*1*) Che205, (*2*) Che667, (*3*) Si112, (*4*) Si82, (*5*) Si199, (*6*) Si84, (*7*) Si199, (*8*) Che642, (C1) *B. subtilis* 652, and (C2) *E. coli* JM103. Panel (b): strains (*1*) Che205, (*2*) Che667, (*3*) Si112, (*4*) Si82, (*5*) Si199, (*6*) Si84, (*7*) Si199, (*8*) Che642, (C1) *B. subtilis* 652, (C2) *E. coli* JM103. Panel (c): Identification of Baikal strains by PCR–genomic fingerprinting. Lanes *1–4* show PCR with the primer N1 and *5–8*, PCR with the primer N3. Strains: (*1* and *5*) Che1008, (*2* and *6*) Che1012, (*3* and *7*) Che1015, and (*4* and *8*) LB-40; M, molecular weight markers.

The overall characteristics of Baikal strains of microorganisms obtained based on morphological and biochemical properties and chromosomal DNA analysis

Groups of strains (genomic fingerprinting)	Strain	Cell morphology and spore production				Mol % G+C	Genera and species of strains close in DNA G+C composition [1]	Growth temperature (°C)		
		Cell shape	Gram staining	Endospores	Swelling of sporangium			30	42	50
Ia	LB-39	Bacilli	+	ETC	–	47.8	*Bacillus coagulans* (*licheniformis*, and *polimyxa*)	++	++++	++++
	LB-44	″	+	ET	+	49.0	*B. coagulans* (*licheniformis*, *polimyxa*, and *macerans*)	++	++++	++++
	LB-40	″	+	ETC	–	45.2	*B. subtilis* (*coagulans, polimyxa*, and *pumilis*)	++	++++	++++
	LB-59	″	+	ET	–	45.9	*B. subtilis* (*licheniformis*, and *polimyxa*)	++	++++	++++
	LB-175	″	+	ETC	±	46.1	″	++	++++	++++
	LB-176	″	+	ETC	±	46.1	″	++	++++	++++
Ib	Che1008	″	+	ET	–	49.0	*B. coagulans* (*licheniformis, polimyxa*, and *macerans*)	++++	++++	++++
	Che1012	″	+	ET	–	49.4	″	++	++++	++++
	Che1015	″	+	ET	–	48.0	*B. coagulans* (*licheniformis*, and *polimyxa*)	++++	++++	++++
	LB-8	″	+	ETC	–			++	++++	++++
	LB-194	″	+	ETC	–			++	++++	++++
	LB-41	″	+	ET	–	45.2	*B. subtilis* (*coagulans, polimyxa*, and *pumilis*)	++	++++	++++
II	LB-43	″	+	ETC	+	50.5	*B. polimyxa* (*coagulans*)	++	++	–
	LB-182	″	+	ET	+	50.6	″	++	++	–
III	Che642	″	+	ET	–			++	++	–
	Che667	″	+	ET	–			++++	++++	–
IV	Si80	″	–	–	–	63.4	Genus *Pseudomonas*	+++	+++	–
	Si82	″	–	–	–	63.0	″	+++	+++	–
	Si84	″	–	–	–	63.2	″	+++	+++	–
	Si112	″	–	–	–	63.6	″	+++	+++	–
V	Si199	Cocco-bacilli	v	–	–	44.6	Genus *Acinetobacter*	+	+	–
	Che205	″	v	–	–	43.8	″	+++	+++	–
VI	LB-56	Bacilli	–	ETC	+	50.4	*B. polimyxa* (*coagulans*)	±	+	–
VII	LB-60	″	+	ETC	–	45.0	*B. subtilis* (*licheniformis*, *polimyxa*, *brevis*)	+	++++	–
VIII	LB-62	″	+	ET	–	–	–	+	++	–
IX	LB-28	″	+	ETC	–	35.7	*Bacillus cereus*	+	++	–
	Che1065	″	+	ET	–	35.7	″	+++	++	±
	Che803	″	+	ET	+	38.3	*B. cereus* (*megaterium*)	++	±	–
X	LB31	″	+	ETC	–			+++	+++	++
XI	Che1010	″	+	ET	–	43.2	*B. subtilis* (*licheniformis*, *polimyxa*, *brevis*, *nutto*)	+++	++	±
XII	Si103	Cocco-bacilli	v	–	–	63.7	Genus *Micrococcus*	+	–	–

Note: In the description of spore production, E stands for elliptic spores; T, for terminal location of spores; and C, for central location. (+) indicates the presence of trait; (±), its weak manifestation; and (–), the absence of trait. The growth intensity at various temperatures is indicated as (–) when the growth is absent; (±) in the case of a trace growth; (+), occurrence of growth; (++), good growth; (+++), active growth; (++++), abundant growth, and v – variable staining.

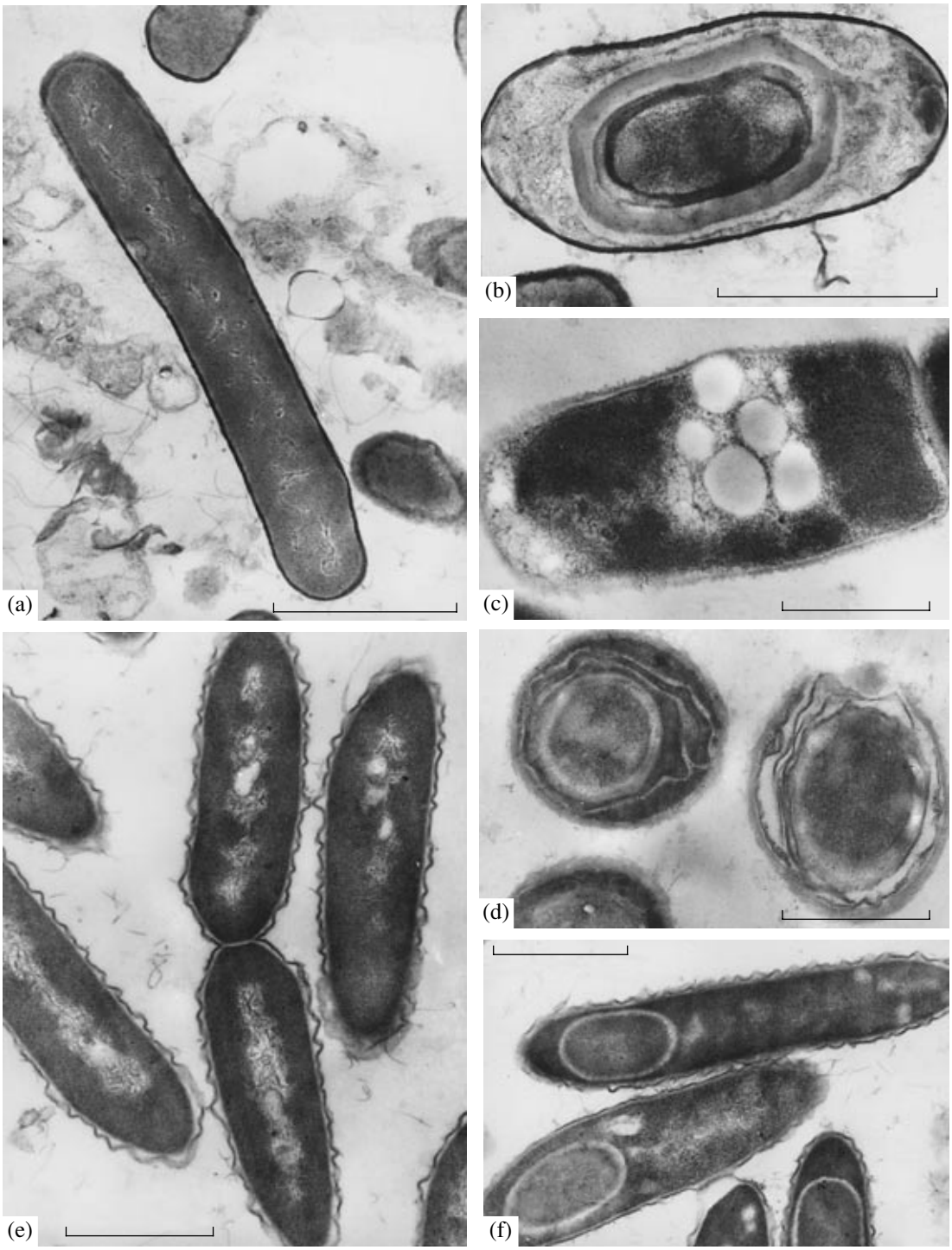

Fig. 2. Ultrathin sections of microbial cells. The line segment in all photographs corresponds to 1 μm. (a) A vegetative cell of the strain LB-194 (subgroup A); electron-dense membrane and nucleoid material are seen. (b) A spore surrounded by the multilayer membrane in the cell of Che1008 strain (subgroup A). (c) A vegetative cell of the strain LB-28 (subgroup B); fatlike inclusions in the cytoplasm are seen. (d) Spores of the strain LB-28, surrounded by the multilayer membrane (subgroup B). (e) Vegetative cells of the strain LB-56 (subgroup C); outer and inner membranes and expanded periplasmic space are seen. (f) Spores in the cells of strain LB-56 (subgroup C).

the fingerprint with the primer N3—we considered it appropriate to separate these strains into an independent subgroup, Ib. The strain LB-41 displayed a similar pattern of PCR fragments with the primer N3.

Phenotypic characteristics of the strains. The strains under study were divided into three major groups according to their morphophysiological characteristics, namely, gram-negative, non-spore-forming motile bacilli (Si80, Si82, Si84, and Si112), belonging presumably to the genus *Pseudomonas*; gram-variable nonmotile coccobacilli (Si199, Che205, and Si103); and spore-forming bacteria of the genus *Bacillus* (table).

According to genomic fingerprinting (GF) data, the strains Si80, Si82, Si84, and Si112 constitute group IV. All four microorganisms display amylase, oxidase, and catalase activities: lack proteolytic activity; do not form acid and gas from sugars; induce hemolysis; are negative in the Voges–Proskauer test and reaction with methyl red; grow on media with 5% NaCl; and are resistant to the same 11 antibiotics. The strains have insignificant differences in morphology.

The gram-variable strains Si199 and Che205, which display spherical or coccobacillary cells, are nonmotile, and lack endospores, were combined into group V, according to GF patterns. Despite morphological similarity and close values of G+C compositions of their DNA, these strains are rather distinct in their biochemical characteristics and antibiotic resistances. The strain Si103, which also has spherical cells, differs in both GF pattern and phenotypic characteristics from the cocci described above. Consequently, it was classified in group XII, according to the GF data. Except for the presence of catalase and oxidase activities, the strain is negative in all the tests performed and fails to produce acid and gas from sugars.

Analysis of the nucleotide composition of chromosomal DNA (table) demonstrated that the DNA of strains Si80, Si82, Si84, and Si112 contained 63.1–63.7 mol % G+C, thereby supporting their attribution to the genus *Pseudomonas.* The G+C content of the strains LB-28 and Che1065 amounted to 35.7 mol %, coinciding with the G+C composition of *B cereus.* Although similar nucleotide composition is a necessary condition for genetic kinship of the microorganisms compared, it is not sufficient. These strains and the closely related strain Che803 (38.3 mol % G+C) were plated onto the Mossel selective medium [13] intended for isolating *B. cereus.* Only the strain Che1065 displayed the growth pattern characteristic of this species, and, for this strain only, species attribution according to other phenotypic traits was confirmed. The strains of bacilli LB-40 and LB-41 contained 45.2 mol % G+C, and LB-175 and LB-176 contained 46.1 mol % G+C. The rest of the strains represented a group containing 43.2–50.5 mol % G+C in their DNA.

Ultrastructural examination of the heterogeneous group of bacillus strains allowed us to separate several subgroups of with similar cellular structures (A, B, and C). Subgroup A comprises bacillary strains Che1008, Che1010, Che1012, LB-23, LB-31, LB-40, LB-41, LB-44, LB-59, LB-60, LB-175, LB-176, LB-194, LB-39. All these microorganisms (Fig. 2a) have the shape of rods with rounded ends and are covered with a single cell membrane, which contains osmiophilic material that produces the visual effect of a dense and thick envelope. The cytoplasm is homogeneous and fine grained. As a rule, the mature endospores are encompassed by a multilayer membrane (Fig. 2b), which exceeds the cell diameter in a number of strains.

Subgroup B contains the strains LB-28 and Che1065, represented by thickened rodlike cells with blunt ends displaying a gram-positive type of structure; the cells are either solitary, paired, or form chains (Fig. 2c). The presence in the cell cytoplasm of large fatlike drops lacking membranes and with a low electron density is a specific feature of this strain. The spores are oval and do not exceed the cell diameter (Fig. 2d).

The strain *Bacillus species* LB-56 (subgroup C) is represented by rod-shaped cells, produces endospores, is gram-negative as determined by the staining, and has a positive reaction with KOH. A fine structure of the cell wall characteristic of gram-negative bacteria is evident in the ultrathin section (outer and inner membranes and a large periplasmic space; Fig. 2e). The spores are terminal; they do not exceed the cell diameter, and are $0.7–1.2 \times 0.5–0.75$ μm in size. In the sections, the spores are surrounded by a thin rim of cytoplasm and the cell membrane (Fig. 2f). The specific morphological features and biochemical characteristics did not allow us to identify the strain *Bacillus* sp. LB-56 as *B. polymyxa* or *B. coagulans*, despite the similarity in DNA nucleotide composition (table). Examples of gram-negative bacilli, e.g., *Metabacterium* sp. [14, 15], are described in the literature; however, such strains belong to noncultivated microorganisms. Presumably, additional studies will allow identification of *Bacillus* sp. LB-56 as a representative of a new taxon.

Correlation between the phenotypic characteristics of the bacilli studied and genomic fingerprinting patterns. Based on GF data, the strains constituting the morphologically homogeneous subgroup A were divided into smaller classes, according to the similarity and distinctions of blot hybridization patterns. Within these smaller classes, both similar patterns and specific differences between the strains are observed (table). For example, the microorganisms belonging to subgroups Ia and Ib, as determined by GF, are also closest to each other in a set of phenotypic characteristics. However, the strains of subgroup B LB-28 and Che1065, united into one group (IX) according to GF patterns and displaying the same nucleotide compositions (35.7 mol % G+C), differ in biochemical characteristics.

The strain LB-56, which differs considerably from the rest of the bacilli studied in its phenotypic traits, has no microorganisms with a similar blot hybridization pattern and is ascribed to a separate group, group VI. This strain displays oxidase and catalase activities, hydrolyzes casein, and is negative in all the other tests. It is resistant to 6 out of the 17 antibiotics tested, namely, to levomycetin, carbenicillin, lincomycin, ristomycin, rifampicin, and sulfathiazole, whereas the rest of the bacilli are sensitive to these antibiotics.

The strains LB-60 and LB-62, representing separate groups (VII and VIII, respectively) according to GF data, display similar morphologies and types of sporogenesis; however, they are essentially different in biochemical characteristics. Moreover, multiple resistance to antibiotics (to 10 out of the 17 tested) is characteristic of the strain LB-62.

Thus, the genomic polymorphism of bacterial strains isolated from sediments of Lake Baikal was studied using genetic fingerprinting methods. The experiments performed allowed us, based on blot hybridization patterns, to divide all the microorganisms studied into several groups reflecting the degree of homology between their genomic DNA. The strains were also characterized phenotypically.

It is demonstrated that the identification of the microorganisms in question, based on morphological, biochemical, and ultrastructural characteristics, complies, to a considerable extent, with the results of genomic analysis. In the case of discrepancies, we may speak with a high probability about the discovery of new taxa of microorganisms.

ACKNOWLEDGMENTS

The work was supported by DOE-IPP (grant no. 6480540) and ISTC (grant no. 2490p).

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