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EXPERIMENTAL ARTICLES

The New Eubacterium *Roseomonas baikalica* sp. nov. Isolated from Core Samples Collected by Deep-Hole Drilling of the Bottom of Lake Baikal

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Abstract—Microbiological analysis of samples of sedimentary rocks from various eras of the geological history of the Baikal rift has enabled us to isolate a large number of microorganisms that can be classified into new, previously undescribed species. The present work deals with the identification and study of the morphological, biochemical, and physiological properties of one such strain, Che 82, isolated from sample C-29 of 3.4–3.5 Ma-old sedimentary rocks taken at a drilling depth of 146.74 m. As a result of our investigations, strain Che 82 is described as a new bacterial species, *Roseomonas baikalica* sp. nov., belonging to the genus *Roseomonas* within the family *Methylobacteriaceae*, class *Alphaproteobacteria.*

Key words: novel microorganism, phenotypic and genomic analysis, Lake Baikal, sedimentary rocks.

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The microorganisms of the Baikal rift are of special interest due to the antiquity of Lake Baikal and the isolated nature of the evolutionary events that occurred there. With drilling equipment, samples of ancient sedimentary rocks represented by silts formed at an average speed of 4 cm/1000 years have been collected. These silts contain unique paleoinformation presenting a continuous record of climatic and biocenotic changes in Central Asia over the past 20–30 million years.

A great number of viable microbial colony-forming units (up to 10^3–10^6 CFU/g) have been detected in the studied samples; more than 2200 microbial isolates have been obtained. Among them, bacteria of the genera *Micrococcus*, *Staphylococcus*, *Bacillus*, *Nocardia*, etc., are abundant; actinomycetes, yeasts, and mold fungi have also been detected. Preliminary study of the taxonomic position of the microorganisms inhabiting the deep horizons of the bottom sediments by fatty acid analysis (FAA) showed that the isolated bacteria belonged to 17 genera, including *Acinetobacter, Arthrobacter, Rhodococcus, Cutrobacterium, Clavibacter*, etc. According to the results of FAA, over 40% of the aerobic bacteria recovered from the bottom sediments were unknown and not deposited in any microbial strain database [1–3]. To construct a phylogenetic tree, we used the nucleotide sequences of 16S rRNA genes from the chromosomal DNA determined by us for some microorganisms from the deep bottom sediments of Lake Baikal, along with the closest homologous sequences from genome databases.

This work is devoted to the description and identification of the bacterial isolate Che 82, classified as a new species, *Roseomonas baikalica* sp. nov.

MATERIALS AND METHODS

Sampling area. Bottom sediments were sampled in 1999 from a well drilled within the framework of the international Baikal Drilling Project (52° 05′ 27.1″ N; 105° 50′ 23.5″ W). The samples for microbiological analyses were aseptically collected from the central part of the core. Sedimentary rocks generally included clays, aleuritic and diatomaceous silts, and diatomaceous shell sediments.

To control the extent of the sample contamination with the drilling agent, we used 1.0-μm polysty-

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rene latex surfactant-free microspheres (Interfacial Dynamic Corp., Portland, Oregon, United States), according to the scheme described in [4].

Isolation of pure cultures and determination of their physiological and biochemical properties were carried out by standard methods [5, 6]. The morphology of the strains obtained was investigated by light microscopy of living and stained cells, as well as by electron microscopy (Hitachi H-600, Japan) of ultrathin sections. The samples were incubated on a shaker (KT 104, Russia) under appropriate temperature conditions in order to ensure growth and biomass production.

Determination of the DNA G+C content. The high-molecular-weight DNA from microbial cells was extracted using the Marmur method [7]. The nucleotide composition of DNA was determined by the thermal denaturation method in 0.1× SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) on a spectrophotometer (heating rate of 0.5 °/min). The DNA melting was carried out on a Milichrom chromatograph equipped with a UV detector and a special cuvette. The G+C content was calculated using the formula (G+C = 2.08 × T_{melt} – 106.4) adapted for low-ionic-strength solutions. The DNA of *E. coli* B with the known nucleotide composition was used as an internal standard.

Sequencing of PCR products corresponding to the 16S rRNA gene fragments. The total DNA of the pure culture of the Che 82 strain was extracted by lysis of precipitated bacterial cells in 4 M guanidine isothiocyanate and subsequent deproteinization with a phenol : chloroform mixture (pH 8.0) and precipitation with isopropanol [8]. PCR analysis was performed in a Tercyc PCR Cycler (DNA-Technology, Moscow, Russia) using universal primers F1 (forward) 5'-AGAGTTTGATCCTGGCTCAG-3') (positions 1–20 on the 16S rRNA gene of *E. coli* DQ360844) and R1 (reverse) 5'-CGGCTACCTTGTTACGACTT-3' (positions 1483–1502 on the 16S rRNA gene of *E. coli* DQ360844); F2 (forward) 5'-GTGCCAGCAGCCGCGGTAA-3' (positions 508–526 on the 16S rRNA gene of *E. coli* DQ360844) and R2 (reverse) 5'-GACGGGCGGTGTGTACAAG-3' (positions 1380–1398 on the 16S rRNA gene of *E. coli* DQ360844), corresponding to the eubacterial 16S rRNA gene; as well as the primers F3 (forward) 5'-GATTAGATACTGTGCATGTAGT-3' (positions 722–743 on the 16S rRNA gene of Che 82) and R3 (reverse) 5'-ACGTGCTGGCAACTAAAGG-3' (positions 1045–1063 on the 16S rRNA gene of Che 82), corresponding to the internal site of the 16S rRNA gene of *Roseomonas*. The following regime was chosen: 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; 30 cycles.

To determine the nucleotide sequences by Sanger's method, the PCR products were purified by gel filtration in a microcolumn (30 mm × 7 mm) filled with Sephadex G-100 (Pharmacia, Sweden). The reaction mixture (8.5 μl) contained 200–600 fmoles of the purified PCR product, one pmol of the relevant primer, and 3 μl of the BigDye 3.1 Terminator Cycle Sequencing Kit mixture (Applied Biosystems, United States). The Sanger reaction was performed in a GeneAmp PCR System 9700 amplifier (PE Applied Biosystems) using the following program: 96°C for 20 s, 96°C for 10 s, 64°C for 4 min, 2 cycles; 96°C for 10 s, 60°C for 4 min, 4 cycles; 96°C for 10 s, 50°C for 7 s, 64°C for 4 min, 12 cycles; 96°C for 10 s, 60°C for 4 min, 4 cycles; and 96°C for 10 s, 50°C for 7 s, 60°C for 4 s, 12 cycles. The removal of the excessive fluorescently labeled dideoxynucleoside-5'-triphosphates was also performed by gel filtration in a microcolumn (30 mm × 7 mm) filled with Sephadex™ G-50 Fine DNA grade (Amersham Biosciences, Sweden). The products of the Sanger reaction were analyzed with an ABI 310 automatic DNA analyzer (Applied Biosystems, United States). The nucleotide sequence of the 16S rRNA gene of strain Che 82 was deposited in the GenBank database (accession number DQ508813).

The phylogenetic analysis of the nucleotide sequence of strain Che 82 and of the nucleotide sequences of 16S rRNA genes from the GenBank database (http://www.ncbi.nlm.nih.gov/) was performed using the MEGA v 3.1 software package [9].

The fatty acid analysis was performed according to the recommendations of MIDI (Newark, United States) [10]. Strain Che 82 was grown on the agarized nutrient medium Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, United States) through three transfers. From the agar surface, a total of 50 mg of culture was harvested and extracted. The fatty acid profile was determined on an Agilent Technologies Model 6890 gas chromatograph (Palo Alto, CA). The obtained chromatograms were analyzed using the Sherlock Microbial identification System Version 4.5 software package (MIDI, Newark, DE, United States).

RESULTS AND DISCUSSION

Strain Che 82 was isolated from sample C-29 incubated on LB medium [5] (pH 7.0–7.2) at 26–28°C. The sample was taken at a drilling depth of 146.74 m; the age of sedimentary rocks was 3.4–3.5 Ma.

Morphological properties of strain Che 82. On LB medium, the strain forms light pink shining semitransparent confluent mucoid even-edged colonies (Fig. 1e) that do not absorb UV radiation. The cells of the day-old culture are motile, spherical or ovoid, and appear in pairs or separately; they are gram-negative, do not produce endospores, and form capsules. Morphological studies of strain Che 82 cells at the stage of exponential growth by phase contrast microscopy have revealed the presence of polymorphic motile cells 1.3–1.8 μm or more in diameter; many of them were in the process of binary division (Fig. 1c). Ultrastructural observations revealed spherical cells (Fig. 1a) surrounded by a double membrane, the outer layer of

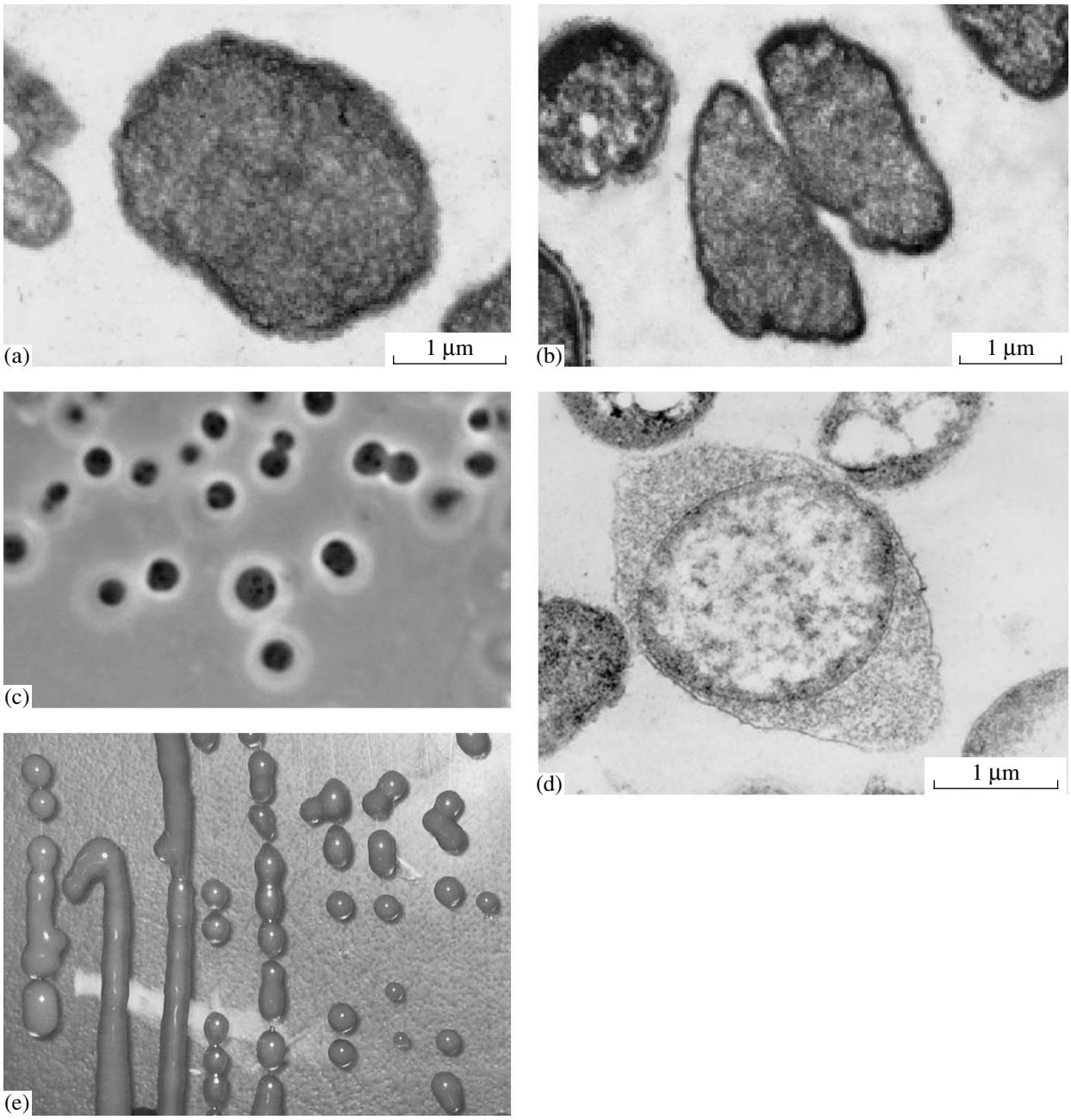

Fig. 1. Morphology of strain Che 82 cells and colonies. (a) Single cell; the cytoplasm is filled with a granular substance; the surface is covered with thin fibrillar material; (b) two daughter cells at the last stage of division. Electron microscopy. The scale bar, 0.5 μm; (c) phase contrast image of dividing cells of a one-day culture; (d) large inclusion in the periplasm of an old Che 82 cell; (e) pink confluent Che 82 colonies on the agarized medium.

which is covered with thin short fibrils. An electron-dense homogeneous substance is contained between the outer and inner layers of the membrane, which hampers the membrane visualization. The cytoplasm is filled with a granular substance of medium electron density; the nucleoid is undetectable. The cells divide by constriction; the daughter cells, which remain connected after division, resemble a butterfly (Fig. 1b). In old cultures, the cell shapes change; cells become polygonal; many of them become more transparent and increase in size up to 1.5–1.8 μm or more; the periplasmic space expands; the two membranes become well defined (Fig. 1d).

Biochemical properties of strain Che 82. Strain Che 82 does not ferment carbohydrates with the resulting formation of acid and gas. The Voges–Proskauer and methyl red reactions are negative; it does not show amylase, lipase, gelatinase, lecithinase, or phosphatase

Table 1. Morphological and biochemical properties of strain Che 82

Property	Strain characteristics	Property	Strain characteristics
Cell shape and size (μm)	Spherical; diameter varies from 1.3 to 1.8 or more	Nitrate reduction	+
Casein hydrolysis	+	Sensitivity to penicillin	Pc^R (30 μg/ml)
Motility	+	Indole production	–
Endospores	–	NH_3	+
Gram reaction	–	H_2S	–
G + C content, mol %	68.1	Citrate utilization	–
Colony morphology	Pink shining semi-transparent confluent	Acid and gas production from carbohydrates	
		Starch	–
Enzymatic activity		Glycerol	–
Catalase	+	Glucose	–
Oxidase	+	Sucrose	–
Urease	+	Xylose	–
Amylase	–	Arabinose	–
Gelatinase	–	Galactose	–
Lipase	–	Lactose	–
Lecithinase	–	Sorbitol	–
Endonucleases	+	Maltose	–
Phosphatases	–	Mannitol	–
Growth at pH 5.4	+	Methyl red reaction	–
Voges-Proskauer reaction	–	Hemolysis on blood agar	–
Growth on a complete medium with 1% methanol	+	Growth on a minimum medium with 1% methanol	–

Note: "+," positive; "–," negative.

activities. These microorganisms do not utilize citrate; they do not produce indole and hydrogen sulfide; when grown on blood agar, they do not form hemolysis zones; methanol is not utilized; acetamide is not assimilated. Bacteria of strain Che 82 hydrolyze casein and exhibit positive reactions for catalase, oxidase, and urease; they produce ammonia when growing on complete medium and cannot grow at high salt concentrations (7% NaCl). Microorganisms of strain Che 82 have arginine, ornithine, and lysine decarboxylases, which is unusual for the known species of the genus *Roseomonas*. Strain Che 82 grows at 25, 30, 37, and 42°C, at pH 5.4, and penicillin concentration of 30 μg/ml. It is sensitive to the following antibiotics: imipenem (0.5 μg/ml), tetracycline (10 μg/ml), tobramycin (2 μg/ml), amikacin (0.25 μg/ml), and ciprofloxacin (0.5 μg/ml) (Table 2).

The above-mentioned properties of strain Che 82 conform with the major characteristics of the representatives of the genus *Roseomonas* (Tables 1–3) [3, 11, 12]. All strains of this genus grow on 5% blood and chocolate agar media, as well as on the agarized BCYE, TSA, and MacConkey media [12]. Most *Roseomonas* strains are resistant to a wide range of penicillins, but may be sensitive to those containing β-lactamase inhibitors (amox/clavulanate and ticar/clavulanate); they are highly sensitive to such antibiotics as imipenem, tetracycline, tobramycin, amikacin, ciprofloxacin, and norfloxacin.

The content of G+C base pairs in Che 82 is 68.1%, which is not beyond the range of values characteristic of *Roseomonas* species.

Hence, according to its morphological and biochemical properties, strain Che 82 belongs to the family *Methylobacteriaceae* and genus *Roseomonas* [11]. The phenotypic properties of strain Che 82 are different from those of the previously described species of this genus, represented mainly by clinical isolates (Tables 1–3) [11–13].

Classification of bacteria based on comparative analysis of 16S rRNA gene sequences allowed the classification of the studied bacterial species into vari-

Table 2. Main distinctive biochemical characteristics of the genera *Methylobacterium* and *Roseomonas* in comparison with those of strain Che 82

Test	*Methylobacterium* spp.	*Roseomonas*** spp.	Che 82
Oxidase	+	+	+
Methanol oxidation	+	–	–
Growth			
on MacConkey agar	–	+	+
at 42°C	–	+	+
Urease	+	+	+
UV absorption by colonies*	+	–	–
Acetamide assimilation	+	–	–
Colony morphology	Dry, coral	Mucoid, light pink	Mucoid, light pink
Cell morphology	Vacuolated rods	Short rods, cocci	Coccobacilli

Note: * Colonies darken under long-wave UV irradiation; ** data presented in [20].

Table 3. Biochemical properties of the pink-pigmented genomospecies of the genus *Roseomonas* and strain Che 82

Test strain/property	Che 82	*Roseomonas roseus*	*Roseomonas ilardii*	*Roseomonas cervicalis*	*Roseomonas fauriae*	*Roseomonas* genomospecies 4	*Roseomonas* genomospecies 5	*Roseomonas* genomospecies 6
Esculin	–	±	–	–	+	–	–	+
Glycerol	–	±	+	–	+	–	±	+
D-mannose	–	±	±	–	–	+	–	–
L-arabinose	–	±	±	±	+	±	±	–
Citrate	–	±	+	±	±	–	±	+
Fructose	–	±	+	+	+	±	+	+
D-galactose	–	±	±	–	+	±	±	–
D-glucose	–	±	±	–	±	–	–	–
MacConkey agar	+	+	+	+	+		+	+
D-mannitol	–	±	±	–	–	–	–	–
Motility	+	+	±	+	+	±	–	+
Nitrate reduction	+	±	±	–	+	+	–	+
D-xylose	–	±	±	±	+	+	±	–
DNA G + C, mol %	68.1	65–71	67.6–71.2	67.5–70.4	68	67.8	65.0	65.4

Note: "+," positive; "–," negative; "±," variable.

ous taxonomic groups. At present, this approach is one of the main methods for the study of the biodiversity and phylogenetic relationships between members of bacterial communities in various ecosystems [14–18].

Comparative analysis of the nucleotide sequence of the 16S rRNA gene fragment (1442 bp) of strain Che 82 revealed its similarity with the known species of the genus *Roseomonas* (90–99% homology). The highest similarity (99%) was observed with the *Roseomonas* strains of genomospecies 4 ATCC 49959 (GenBank accession number AF533355), ATCC 49959 (AY150048), and JS018 (DQ010108) isolated in China. According to a phylogenetic analysis of the 16S rRNA gene sequences, strain Che 82 belongs to the genus *Roseomonas*. However, it certainly differs from all the previously described species of this genus (Fig. 2). The phylogenetic tree constructed with the neighbor-joining method (Fig. 2) illustrates the reliability of strain Che 82 species identification. Phylogenetic trees constructed with alternative algorithms of the maximal parsimony, minimal evolution, and UPGMA have a similar topologies (data not presented).

According to the results of the fatty acid analysis, strain Che 82 belongs to the genus *Roseomonas*; due to the small convergence coefficient (0.489, Table 4), it cannot be affiliated with the previously described species *R. cervicalis, R. fauriae, Roseomonas* genomospecies 4, 5, 6, etc. The composition and proportion of fatty acids in strain Che 82 also differ from those in the

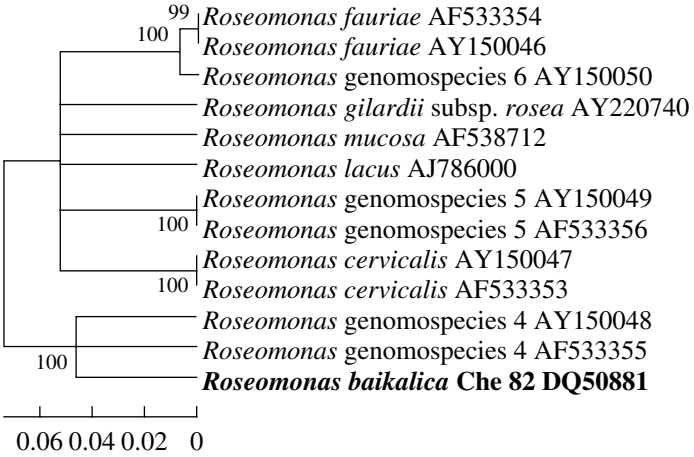

Fig. 2. Phylogenetic tree of the 16S rRNA gene fragment sequences of eubacteria of the genus *Roseomonas* constructed with the neighbor-joining method. The names of eubacterial species and their accession numbers in the GenBank database are presented. Numerals at the branching points indicate the support indices of cladistic groups. The sequence in bold belongs to the studied strain Che 82.

recently described strain *R. lacus*, isolated from the bottom sediments of a freshwater lake in China [19].

The results of the complex genomic and phenotypic analysis of strain Che 82 allowed us to affiliate this strain to the genus *Roseomonas* as a new species *Roseomonas baikalica* sp. nov. (I.S. Andreeva et al., 2006). The species was named after Lake Baikal where it was isolated.

Description of *Roseomonas baikalica* sp. nov. *Roseomonas baikalica* sp. nov., isolated from the sample C-29 incubated on LB medium (pH 7.0–7.2), produces light-pink, shining, semi-transparent confluent mucoid even-edged colonies that do not absorb UV radiation. The cells of the day-old culture are motile and either spherical or ovoid in shape; they appear in pairs or separately; they are gram-negative, do not produce endospores, and form capsules. The cell size varies from 1.31 to 1.8 μm or more; the cells reproduce by binary division.

Table 4. Fatty acid profile of strain Che 82

Fatty acid	%*
12:0	0.38
14:0	0.46
14:0 3OH/16:1 *iso* 1	0.34
16:1 ω7c/15 *iso* 2OH	13.83
16:1 ω5c	3.95
16:0	7.75
17:0 ω6c	2.04
16:0 3OH	0.99
18:1 ω7c	59.60
19:0 *cyclo* ω8c	0.83
18:1 2OH	9.83

Note: * The percentage of the total fatty acid concentration for each fatty acid.

R. baikalica sp. nov. does not ferment carbohydrates with the resulting formation of acid and gas. It is Voges–Proskauer and methyl red negative; it does not have amylase, lipase, gelatinase, lecithinase, or phosphatase activities; does not utilize citrate; does not produce indole or hydrogen sulfide; when grown on blood agar, it does not form hemolysis zones; and it is able to hydrolyze casein. Bacteria of this species are positive for catalase, oxidase, and urease; they produce ammonia when growing on complete medium and have nuclease activity; they grow at pH 5.4 and penicillin concentration of 30 μg/ml, as well as on MacConkey media. The species has arginine, ornithine, and lysine decarboxylases. It does not utilize methanol and does not assimilate acetamide; it grows at 25, 30, 37, and 42°C. The species cannot grow at high salt concentrations (5.7% NaCl). The DNA G+C content of *R. baikalica* sp. nov. is 68.1 mol. %.

The type strain *R. baikalica* Che82 is deposited with the strain collection of the Vektor State Research Center of Virology and Biotechnology (accession number B-1054).

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