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EXPERIMENTAL ARTICLES

Heliobacterium sulfidophilum sp. nov. and *Heliobacterium undosum* sp. nov.: Sulfide-oxidizing Heliobacteria from Thermal Sulfidic Springs

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Abstract—Two new species of heliobacteria isolated from cyanobacterial mats of two alkaline sulfidic hot springs are formally described. Strains BR4 and BG29 are assigned to anoxygenic phototrophic bacteria of the family *Heliobacteriaceae*, since they possess the unique properties of this taxon: strict anaerobiosis, formation of bacteriochlorophyll *g*, the lack of extensive intracytoplasmic membranes and chlorosomes, an unusual cell wall structure, and phylogenetic relatedness to the low G+C gram-positive eubacteria. Based on the 16S rDNA sequence similarity, strains BR4 and BG29 are assigned to the genus *Heliobacterium* and described as two new species of this genus: *Heliobacterium sulfidophilum* sp. nov. and *Heliobacterium undosum* sp. nov. The G+C content of the DNA is 51.3 mol % in *Hbt. sulfidophilum* and 57.2–57.7 mol % in *Hbt. undosum*. The cells of *Hbt. sulfidophilum* are rods, and the cells of *Hbt. undosum* are slightly twisted spirilla or short rods. Both new bacteria are motile by peritrichous flagella. *Hbt. sulfidophilum* produces endospores. The new bacteria are strict anaerobes growing photoheterotrophically on a limited range of organic compounds. In the dark, they can switch from photosynthesis to the slow fermentation of pyruvate. Biotin is required as a growth factor. Both species are highly tolerant to sulfide (up to 2 mM at pH 7.5) and oxidize it photoheterotrophically to elemental sulfur; photoautotrophic growth was not observed. The temperature optimal for growth of *Hbt. sulfidophilum* and *Hbt undosum* is 30–35°C, and the optimal pH is 7–8.

Key words: anoxygenic phototrophic bacteria, heliobacteria, bacteriochlorophyll *g*, taxonomy, *Heliobacterium sulfidophilum* sp. nov., *Heliobacterium undosum* sp. nov., alkaline thermal springs, phototrophic sulfide oxidation

Heliobacteria are strictly anaerobic anoxygenic phototrophic bacteria containing bacteriochlorophyll *g* as the sole photopigment [1]. They are phylogenetically related to the low G+C gram-positive bacteria, in particular, to the endospore-forming *Bacillus/Clostridium* branch [2]. Paddy soils were long thought to be the only natural habitat of heliobacteria. The samples of rice field soils, therewith often thoroughly dry samples, appear to be an excellent source for the isolation of these bacteria [1]. At present, heliobacteria are known to occur in various aqueous ecosystems. In 1993, for the first time, a mesophilic heliobacterium, strain BR4, was isolated from the cyanobacterial mat of an alkaline sulfide-containing hot spring. Strain BR4 tolerated high concentrations of hydrogen sulfide (up to 2 mM at pH 7.4) and oxidized it to elemental sulfur in the light and in the presence of organic compounds [3]. The new isolate differed from other heliobacteria that were known by that time by a higher pH optimum of 7–8 and a relatively low temperature optimum (30°C). The name "*Heliobacterium sulfidophilum*" was proposed for strain BR4; however, the formal description has not been published [4]. More recently, a number of strains of heliobacteria were isolated from the microbial mats of neutral and alkaline hot springs. These bacteria were described as a new thermophilic species *Heliobacterium modesticaldum* [5]; they had a temperature optimum at 52°C, a pH optimum between 6 and 7, and did not utilize sulfide. By now, two more heliobacteria with a capacity for dissimilatory sulfide oxidation have been described: *Heliorestis daurensis* and "*Heliorestis baculata*" [6, 7]. They were isolated from soda lakes and are obligate alkaliphiles with the pH optimum between 8.5–9.5.

The aim of this work was to determine the taxonomic position of a previously isolated strain BR4 and a new sulfide-oxidizing strain BG29. Both heliobacte-

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ria were isolated from cyanobacterial communities developing in a mesophilic zone of hot springs.

MATERIALS AND METHODS

Isolation sources. Samples for inoculating enrichment cultures of heliobacteria were collected from cyanobacterial mats developing in sulfidic hot springs of Buryatia (Russia). Strain BR4 was isolated from the Bol'sherechenskii spring (pH 9.3; H_2S 10 mg/l; 50°C) located in the Barguzinskii reserve, near the Bol'shaya River, 30 km away from Lake Baikal [3]. Strain BG29 was isolated from a sample collected from the Garginskii spring (pH 8.8; H_2S 1 mg/l; 40°C) located on the side of the Garga River in the Barguzinskaya valley.

Isolation and cultivation. Heliobacteria were enriched and subsequently cultivated using a medium of the following composition (g/l of distilled water): KH_2PO_4, 0.5; NH_4Cl, 0.5; NaCl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.8; $CaCl_2$, 0.1; $NaHCO_3$, 1.5; sodium acetate, 1; yeast extract, 0.1; $Na_2S \cdot 9H_2O$, 0.3; vitamin B_{12}, 20 μg; biotin, 20 μg; and trace elements, 1 ml [8]. The pH was adjusted to 7.6. To control anoxic conditions, resazurin was added to a final concentration of 0.002% (0.2% solution, 1 ml/l).

Pure cultures of heliobacteria were obtained by repeated transfers of colonies from dilution series in 0.8% agar medium of the same composition. The purity of cultures was controlled by microscopy and by transfers on agar medium under aerobic and anaerobic conditions. Liquid cultures of heliobacteria were grown in 30- and 50-ml screw-capped glass bottles or in Hungate tubes at 25–30°C and a light intensity of 2000 lx.

Microscopy. Cell morphology was studied by light microscopy with a phase contrast device and by electron transmission microscopy. Intact cells for electron microscopy were stained with 1% phosphotungstic acid. The ultrastructure of the bacterial cells was studied as described earlier [3].

Analysis of the pigment composition. The pigment composition of the heliobacteria was studied in intact cells suspended in 50% glycerol and in acetone extracts. For in vivo spectra, cells were supplemented with 0.04% sodium thioglycolate to prevent the oxidation of pigments. Spectra were recorded with a LOMO SF 56 spectrophotometer (Russia) in a wavelength range from 350 to 950 nm.

Physiological tests. The ability of heliobacteria to utilize various carbon and energy sources, sulfur and nitrogen compounds, the requirements for vitamins, carbonate and NaCl concentrations, and the pH optimum for growth were evaluated in growth experiments by the biomass increment in the exponential growth phase. The biomass yield was estimated from the culture optical density measured at 650 nm or quantified from the pigment content in acetone–methanol (7 : 2) extracts measured colorimetrically at 660 nm with a KFK-3 photometer (Russia).

The range of utilized organic compounds was determined on basic mineral medium containing 50 mg/l yeast extract as the growth factor. Organic substrates were prepared separately as 5% stock solutions, neutralized, sterilized at 0.5 atm, and added at concentrations of 0.3 or 0.5 g/l.

Sulfur compound utilization and their oxidation products were studied in batch cultures of heliobacteria. Simultaneously present ions $S_2O_3^{2-}$, SO_3^{2-}, and $H_2S + HS^-$ were determined by iodometric titration [9]. Sulfide was measured colorimetrically with *p*-phenylenediamine [10] and sulfur using the modified Morris method [3].

Analysis of the fatty acid composition. Fatty acids and other lipid components were extracted from cell biomass by acid methanolysis. Wet biomass (30 mg) was dried at 80°C in a stream of nitrogen, and 400 μl of a 1 N solution of anhydrous HCl in methanol was added. The mixture was heated at 80°C for 3 h. The resultant methyl esters of fatty acids and other lipid components were extracted with hexane. The extract was evaporated to dryness and silylated in 20 μl of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) for 15 min at 80°C. A 1-μl portion of the reaction mixture was analyzed with a model HP-5973 Hewlett-Packard gas chromatography–mass spectrometry (GC-MC) system equipped with a fused silica capillary column (25 m by 0.25 mm). The quadrupole mass spectrometer has a resolution of 0.5 mass units over the whole range of 2–1000 amu. Ionization is performed by electrons at 70 eV. The sensitivity of the GC–MC system is 0.1 ng of methyl stearate. The temperature profile included a 2-min isotherm at 120°C and subsequent temperature programming at a rate of 5°C/min to 280°C. The injector and interface temperature was 280°C.

Analysis of total cell proteins. Total cell proteins of heliobacteria were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) according to Laemmli [11] with some modifications. A 100-mg portion of wet biomass was washed twice with 0.15 M NaCl solution, supplemented with 100 μl of 0.15 M NaCl, and the cells were disrupted by sonication with a UZDN-2T ultrasound disintegrator (22 kHz) for 1 min at 4°C. The protein concentration in the thus-obtained homogenate was measured by the Lowry method and brought to 1.2–1.4 mg/ml with 0.15 M NaCl solution. The protein suspensions were then mixed with a standard buffer (Tris, 3.1 g; 1 M HCl solution, 20 ml; SDS, 4 g; glycerol, 10 ml; distilled water to a final volume of 100 ml) in the ratio 1 : 1 and boiled for 6 min. The final concentration of the total proteins in the samples was 6–7 μg/μl. The samples were stored at –14°C.

Gel electrophoresis was carried out in a vertical chamber (DESAGA, Germany) at a 40-mA current in the stacking gel and a 60-mA current in the separating gel. From 10 to 20 μl of a sample were loaded into

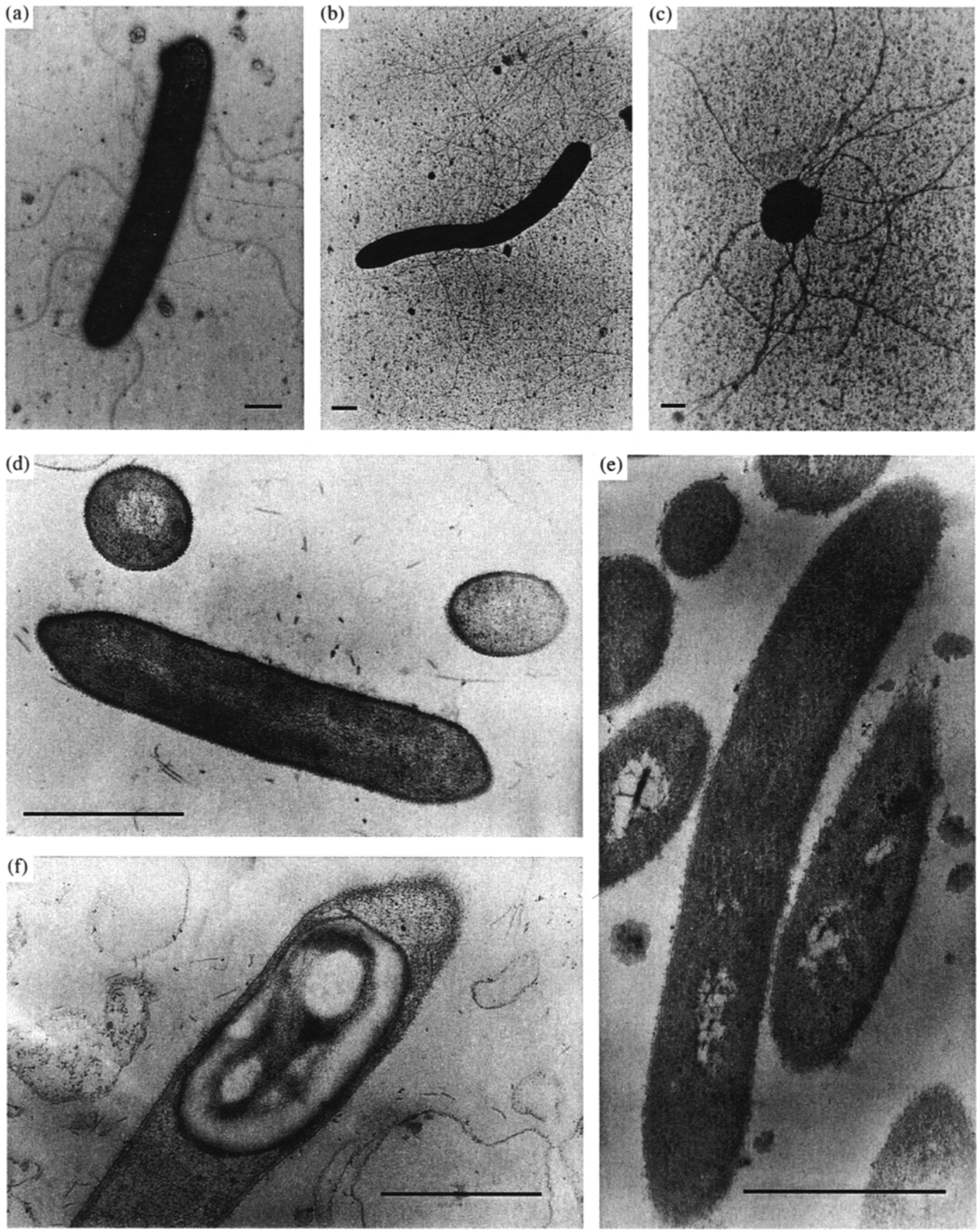

Fig. 1. Morphology and fine structure of heliobacteria: (a), (d), and (f) strain BR4 and (b), (c), and (e) strain BG29. Transmission electron microscope: (a–c) micrographs of whole cells stained with phosphotungstic acid showing the arrangement of flagella on bacterial cells and (c) spheroplasts; (d–f) micrographs of ultrathin sections, (f) shows a spore. Bars represent 1 μm.

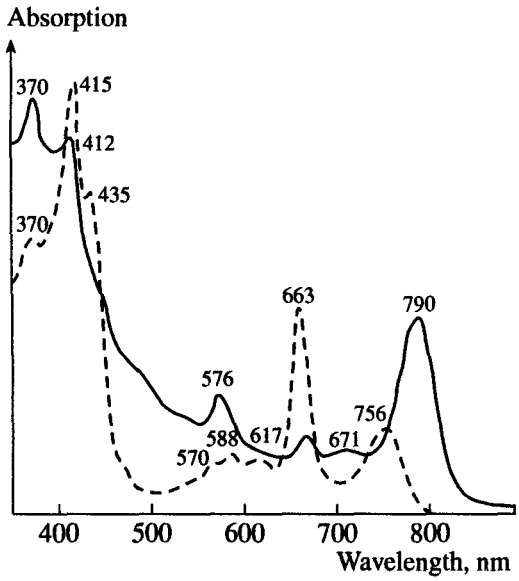

FIg. 2. Absorption spectra of intact cells (solid line) and acetone extract (dotted line) of strain BG29. Absorption spectra of strain BR4 were identical with the spectra of strain BG29, the positions of their maxima differing by no more than 1–3 nm.

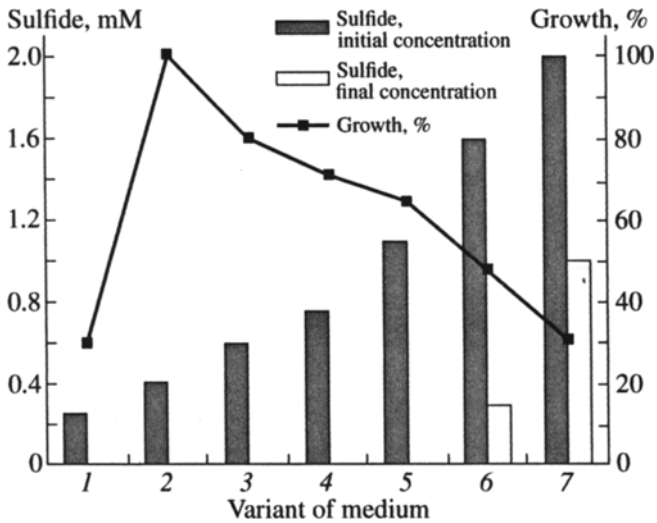

Fig. 3. Sulfide utilization by strain BG29. Seven media were tested differing in their initial sulfide concentrations (mM): (*1*) 0.25; (*2*) 0.4; (*3*) 0.6; (*4*) 0.75; (*5*) 1.1; (*6*) 1.6; (*7*) 2.0.

wells. The following molecular mass standards were used: albumin, 67000 Da; ovalbumin, 45000 Da; myoglobin, 17800 Da; and cytochrome, 12300 Da.

Gels were fixed for 18 h in 50% trichloroacetic acid, stained for 5 h in a 0.01% solution of Coomassi Brilliant Blue R-250, and destained in 7% acetic acid. The total number of bands in the protein spectrum of each strain and the number of protein bands common to both strains compared were calculated. Similarity coefficients were computed from the formula of Dice [12]: $S = (2m/(a + b))100\%$, where m is the number of protein bands common to both strains; a is the number of protein bands occurring with strain 1, and b is the number of protein bands occurring with strain 2. The phenogram was constructed using the original Feno program for processing the experimental results.

Methods of genosystematics. Isolation and purification of DNA, determination of the mol % G+C content in DNA, and DNA–DNA hybridization were performed by the standard methods [13–15]. The 16S rRNA gene was amplified and sequenced as described earlier [16]. Nucleotide sequences of 16S rRNA genes of strains BR4 and BG29 were manually aligned with the respective sequences of the known species of the family *Heliobacteriacea* and several closely related representatives of the phylogenetic branch *Bacillus/Clostridium* obtained from the recent GenBank nucleotide database releases. The positions of sequence and alignment uncertainties were omitted, and a total of 1256 nucleotides were used in the analysis. Unrooted phylogenetic trees were constructed using the programs of the TREECON package [17]. The 16S rRNA gene sequences of strains BR4 and BG29 have been deposited in the GenBank under accession numbers AF 249678 and AF 249679, respectively. The accession numbers of the sequences used as references were as follows: *Heliobacillus mobilis* ATCC 43427^T, L36199; *Heliobacterium chlorum* ATCC 35205^T, M11212; *Heliobacterium gestii* ATCC 43375^T, U14558; *Heliobacterium modesticaldum* ATCC 51547^T, U14559; *Heliophilum fasciatum* ATCC 51790^T, U14557; *Heliorestis daurensis* ATCC 700798^T, AF047462; *"Heliorestis baculata"* OS-H1^T, AF 249680; *Desulfitobacterium dehalogenans* JW/IU-DC1^T, L28946; *Desulfitobacterium frappieri* PCP-1^T, U40078; *Desulfitobacterium chlororespirans* Co23^T, U68528; *Desulfitobacterium hafniense* DCB-2^T, X94975; *Sporotomaculum hydroxybenzoicum* DSM 5475^T, Y14845; *Desulfosporosinus orientis* Singapore I^T, M34417; *Syntrophobotulus glycolicus* ClGlym^T, X99706; and *Peptococcus niger*, X55797.

RESULTS AND DISCUSSION

Habitat. Strains BR4 and BG29 of heliobacteria were isolated from the cyanobacterial mats of the freshwater sulfidic thermal springs of Buryatia. The maximum water temperatures recorded in the springs ranged from 72–78°C. The following hydrochemical characteristics were recorded at the sampling sites: 50°C, pH 9.3, and 10 mg/l sulfide in the spring near the Bol'shaya River; 40°C, pH 8.8, and 1 mg/l sulfide in the Garginskii spring. Microbial communities of the mats included cyanobacteria of the genera *Synechococcus* and *Phormidium*, thermophilic filamentous green bacterium *Chloroflexus aurantiacus* and mesophilic purple nonsulfur bacterium *Rhodopseudomonas palustris*. Pure cul-

tures of heliobacteria were isolated from separate colonies obtained on agar medium incubated at 30°C.

Morphology and fine structure. Cells of strain BR4 were cigar-shaped rods measuring 0.6–1 by 4–7 μm (Fig. 1a). Morphologically, they resembled *Heliobacillus mobilis*. The shape of strain BG29 cells varied from short rods to slightly twisted spirilla resembling *Heliobacterium gestii* cells (Fig. 1b); the cell size also varied in the range 0.8–1.2 by 7–20 μm. Cells of both strains were motile by peritrichous flagella (Figs. 1a–1c). Cells divided by cross-septation. As with other heliobacteria, in the stationary growth phase of strains BR4 and BG29, the formation of spheroplasts and lysis could often be observed [1]. Spheroplasts were often motile due to remaining flagella (Fig. 1c). Strain BR4 formed endospores (Fig. 1f); however, upon long culturing in the laboratory medium, the strain ceased to form endospores. Strain BG29 did not produce endospores.

In both strains, the cell wall structure was typical of other heliobacteria (Figs. 1d–1f). The outer membrane, characteristic of gram-negative bacteria, was not recognized. The peptidoglycan layer was rather thin for gram-positive bacteria. Like all other representatives of the family *Heliobacteriaceae*, strains BR4 and BG29 lacked intracytoplasmic membranes typical of purple bacteria or chlorosomes present in green bacteria.

Pigments. The absorption spectra of whole cells and acetone extracts of strains BR4 and BG29 demonstrated the presence of bacteriochlorophyll *g* (in vivo maximum at 788–790 nm) and of a carotenoid similar in its spectrum characteristics to neurosporene (in vivo peak at 412 nm) [1, 18]. Absorption spectra of intact cells under anaerobic conditions showed peaks at 370–375, 412, 576, 671, and 788–790 nm (Fig. 2). A minor peak at 671 nm belonged to a pigment of the chlorophyll *a* type, which is formed upon the oxidation of bacteriochlorophyll *g* [19]. Absorption spectra of acetone extracts showed maxima at 370, 415, 435, 570, 588, 663, and 756 nm (Fig. 2). The presence of bacteriochlorophyll *g* in the cells of heliobacteria determined the peculiar dark-green color of their colonies and liquid cultures under anaerobic conditions.

Physiological properties. Strains BR4 and BG29 were strict anaerobes. Photoheterotrophy was the main type of metabolism with acetate, pyruvate, lactate, casein hydrolysate, and yeast extract utilized as carbon sources. In addition, strain BR4 could grow on malate and butyrate (+ CO_2), whereas strain BG29 utilized propionate. Both strains were able to grow chemotrophically in the dark at the expense of pyruvate fermentation. Under these conditions, the cells retained their pigmentation. Ammonium, molecular nitrogen, L-glutamine, and casein hydrolysate served as nitrogen sources for strain BR4. Both strains required biotin as a growth factor.

Both strains of heliobacteria were isolated from sulfide-containing springs. The addition of hydrogen sulfide into a medium containing acetate stimulated their

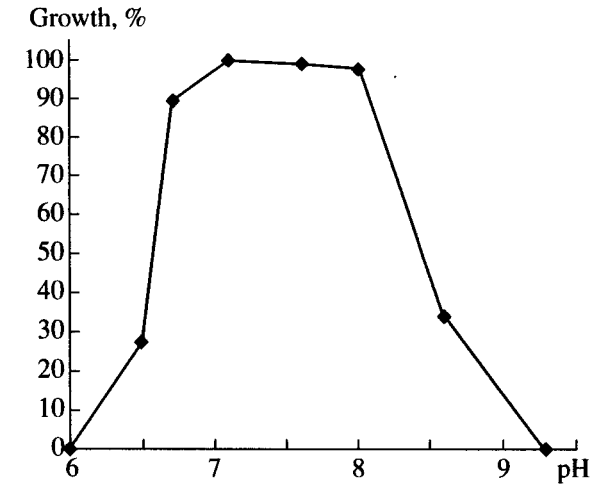

Fig. 4. Influence of pH on the growth of strain BG29.

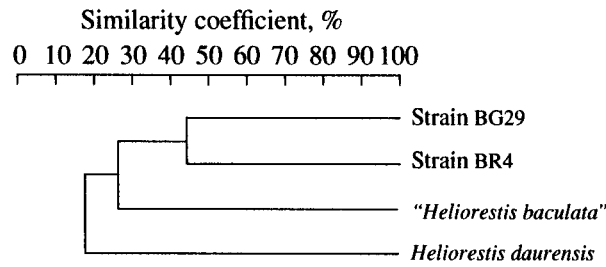

Fig. 5. A phenogram constructed using the similarity coefficients of the total cell proteins in the heliobacteria compared.

growth in the light. At pH 7.5, growth was observed at sulfide concentrations up to 2 mM (Fig. 3). During photosynthesis of strain BR4, sulfide was used as an electron donor oxidized to elemental sulfur as the end product [3]. As with other heliobacteria, there was no evidence of the photoautotrophic growth of either strain. Strain BR4 appeared capable of reducing elemental sulfur to sulfide both in the light and in the dark [3].

Both strains of heliobacteria are freshwater organisms. They ceased to grow at NaCl concentrations approaching 5 g/l. Growth took place over the pH range 6.5–9.0, with the optimum between 7 and 8 (Fig. 4) [3]. The optimum temperature for growth of these strains was 30–35°C, which is some 10°C lower than that for most of the previously described heliobacteria (about 40°C).

Cellular fatty acids. Comparative analysis of fatty acid composition of heliobacteria under study showed that isomers of the fatty acid $C_{17:1}$ containing a single double bond (monoenoic) dominated both in strain BR4 and strain BG29, representing 48.3 and 41.8%, respectively, of the total fatty acids. A significant proportion of the $C_{16:1}$ fatty acid was also found in both strains (28.9 and 27.1%, respectively); this acid was

Table 1. Composition of cellular fatty acids in heliobacteria

Fatty acid*	% of the total fatty acids					
	strain BR4	strain BG29	*Heliobacterium gestii* [20]	*Heliobacterium chlorum* [20]	*Heliorestis daurensis* [7]	*"Heliorestis baculata"* [7]
14 : 1		0.2				
14 : 0	0.2	0.3	0.3	0.6		0.1
*i*15 : 0		0.1	0.5	0.7		
15 : 1Δ7	1.3	1.5	0.2			1.5
15 : 1Δ9	9.5	9.5	0.5			2.4
15 : 0	0.4	1.3	0.1	0.1		2.1
*i*16 : 1Δ7			15			
*i*16 : 1Δ9			7.2	0.1		
*i*16 : 0			1.2	0.2	1.2	
16 : 1Δ7	1.7		2.5	7.1	5.6	3.0
16 : 1Δ9	9.1	13.2	12.1	20.4	17.6	11.7
16 : 1Δ11	18.1	13.9	21.2	18.8	11.4	4.4
16 : 0	2.3	2.5	2.4	8.9	2.7	3.2
*i*17 : 1Δ9		0.6	6.0	3.6		2.9
*i*17 : 1Δ11		0.7	10.2	3.4		
*i*17 : 0	0.7	0.7	1.1	1.5		0.8
17 : 1Δ9	2.4	1.8	1.4	0.5		1.9
17 : 1Δ11	9.0	13.6	4.4	1.2	2.2	11.0
17 : 1Δ13	36.2	25.1	0.5		6.5	25.4
17 : 0	0.3	0.9	0.1	0.1		1.8
*i*18 : 1Δ9			1.7	0.1		
*i*18 : 1Δ11			6.5	0.1		
18 : 2		1.9	3.8	2.0	6.3	
18 : 1Δ7			1.0	0.9	3.1	
18 : 1Δ9	1.7	1.7	2.3	9.4	4.9	5.1
18 : 1Δ11	2.6	2.1	5.3	10.3	31.7	16.9
18 : 1Δ13	2.6	1.9	5.3	7.6	6.1	2.3
18 : 0	1.2	1.6	0.8	1.8	0.7	1.1
19 : 1	0.8	1.0				3.3

* The first two figures show the number of carbon atoms in the chain; the second figure shows the number of double bonds; the third figure shows the position of a double bond; *i*, the branching of the carbon chain. For example, 18 : 1Δ9 designates 9-octadecenoic (oleic) acid.

previously shown to dominate in the fatty acid pattern of other heliobacteria [20]. Only insignificant differences were revealed between the fatty acid compositions of new isolates: low contents of $C_{14:1}$, iC_{15}, $iC_{17:1}$, and $C_{18:2}$ acids were found in strain BG29 but were not revealed in strain BR4. Comparative analysis of fatty acid composition in our new strains of heliobacteria and in a phenotypically related species *Heliobacterium gestii* showed essential qualitative and quantitative differences. *Heliobacterium gestii* was distinguished from strains BR4 and BG29 by the presence of many double isomers of monoenoic acids containing 16, 17, and 18 carbon atoms [20]. Even more essential differences were found between fatty acid patterns of neutrophilic and alkaliphilic representatives of heliobacteria (Table 1) [7].

Total cell proteins. The cell protein compositions of strains BG29 and BR4 were compared with those of previously described alkaliphilic heliobacteria *Heliorestis daurensis*, strain BT-H1^T, and *"Heliorestis baculata,"* strain OS-H1^T [6,7]. The protein spectra of the studied heliobacteria contained 22–30 protein bands, of which number from 2 to 13 similar protein pairs were detected in the protein patterns of the two compared strains. The entire protein spectrum was characterized by R_f values ranging from 0.076 to 0.96. The similarity coefficients for the obtained protein pat-

Table 2. Comparative characterization of heliobacteria

Characteristic	*Heliobacterium undosum*	*Heliobacterium silfidophilum*	*Heliobacterium chlorum* [1]	*Heliobacterium gestii* [1]	*Heliobacterium modesticaldum* [5]	*Heliobacillus mobilis* [1]	*Heliophilum fasciatum* [1]	*Heliorestic daurensis* [6]	*"Heliorestis baculata"* [7]
Morphology	rods, slightly twisted spirilla	rods	rods	spirilla	rods, curved rods	rods	rods in aggregates	wound coils or bent filaments	straight or slightly curved rods
Cell size, μm	0.8–1.2 × 7–20	0.6–1 × 4–7	1 × 7–9	1–1.2 × 5–10	0.8–1 × 2.5–9	1 × 7–10	0.8–1 × 5–8	0.8–1.2 × 20	0.6–1 × 6–10
Endospores	–	+	–	+	+	–	+	+	+
Flagella	peritrichous	peritrichous	absent, gliding movement	polar and subpolar	polar, subpolar, and nonmotile cells	peritrichous	polar and subpolar	peritrichous	peritrichous
Pigments	bchl *g*, carotenoid similar to neurosporene			bchl *g*, 4,4'-diaponeurosporene				bchl *g*, carotenoid similar to neurosporene	
Absorption spectrum in vivo, nm	790, 720(S), 671, 576, 412, 370	788, 670, 575, 412, 375	788, 718(S), 670, 575, 375	788, 718(S), 670, 575, 480, 415, 375	788, 718(S), 670, 575, 430	788, 718(S), 670, 575, 375	792, 723(S), 673, 577, 480, 415	788, 712(S), 671, 576, 405, 370	788, 671, 575, 413, 370
Carbon source	pyruvate, yeast extract, acetate, lactate, casein hydrolysate, propionate	pyruvate, yeast extract, acetate, lactate, casein hydrolysate, butyrate + CO_2, malate	pyruvate, lactate, yeast extract;	pyruvate, yeast extract, acetate, butyrate + CO_2, ethanol* + CO_2, fructose, glucose, ribose	pyruvate, yeast extract, acetate, lactate	pyruvate, yeast extract, acetate, lactate, butyrate + CO_2, ethanol* + CO_2	pyruvate, yeast extract, acetate + CO_2, lactate, butyrate + CO_2, ethanol*	pyruvate, acetate	pyruvate, acetate, lactate
Tolerance to sulfide	up to 2 mM	up to 2 mM	–	up to 1 mM	–	–	up to 0.1 mM	up to 10 mM	up to 3 mM
Sulfide utilization	+	+	–	–	–	–	–	+	+
Growth factors	biotin	biotin	biotin	biotin	biotin	biotin	biotin	biotin	biotin
Optimum temperature for growth, °C	31–36	32	38–42	38–42	52	38–42	37–40	25–35	29–31
Optimum pH for growth	7–8	7–8	6.2–7	7	6–7	6.2–7	7	9	8.5–9.5
G+C content, mol %	57.2–57.7	51.3	52.0	54.5–55.6	54.5–55.0	50.3	51.8	44.9	45
Habitat	alkaline sulfidic hot springs	alkaline sulfidic hot springs	soil	tropic rice field soil	neutral or alkaline hot springs, volcanic soil	tropic rice field soil	rice field soil	microbial mats of brackish soda lakes, alkaline nearshore soil	alkaline nearshore soil of brackish soda lakes

* Trait revealed in occasional strains.

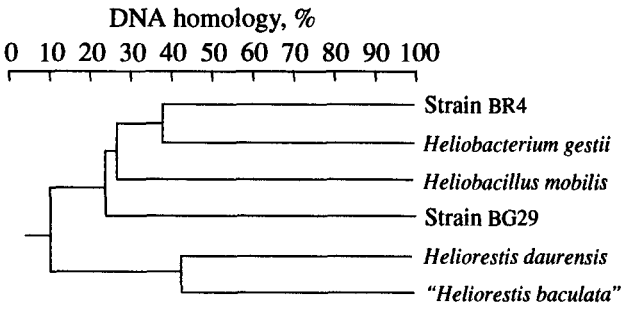

Fig. 6. A dendrogram of phylogenetic relationships between the strains studied and other representatives of the family *Heliobacteriaceae* based on the DNA–DNA hybridization data.

terns, computed from the Dice formula, pointed to a significant distance between the heliobacteria studied. Based on the similarity coefficients, a phenogram was constructed showing the relationships among the heliobacteria (Fig. 5). Strains BG29 and BR4 appeared to be the most closely related, with a similarity level of 43%. These strains significantly differed from the species of the genus *Heliorestis*, the similarity coefficients for them comprising 19–25%. On the other hand, strains BT-H1^T and OS-H1^T, belonging to the same genus *Heliorestis* [7], showed only a 16% similarity between their cell protein patterns.

Genetic properties. The G+C contents of DNA from strains BR4 and BG29 were 51.3 and 57.2–57.7 mol %, respectively. All known heliobacteria except for alkaliphilic species have a similar DNA base composition (Table 2). DNA–DNA hybridization showed that the level of DNA homology between the strains studied was 25% (Fig. 6). Similar DNA homologies were found between strain BR4 and the species *Heliobacillus mobilis* and *Heliobacterium gestii*—25 and 38%, respectively. By the results of DNA–DNA hybridization, alkaliphilic representatives of heliobacteria are rather distantly related to the *Heliobacterium–Heliobacillus* group (5–11%).

Almost complete sequences (more than 1500 nucleotides) of the 16S rRNA genes from strains BR4 and BG29 were determined, which corresponded to positions between 9 and 1520 in *E. coli*. A comparative 16S rRNA sequence analysis confirmed the common origin of all formally described representatives of the family *Heliobacteriaceae*, including the species of the new genus *Heliorestis* (Fig. 6). At the same time, the data of this analysis showed the existence of three distinct phylogenetic clusters separating the family *Heliobacteriaceae* into the genera *Heliobacterium*, *Heliophilum*, and *Heliorestis*. Analysis of the 16S rRNA gene sequences of new strains BR4 and BG29 clustered them together with the species of the genus *Heliobacterium*. However, inside this cluster, they form separate branches essentially differing from the known species and also from each other (at most, a 98% similarity). Similarity with the most closely related representatives of *Bacillus–Clostridium* branch did not exceed 86.6%.

Taxonomic position. The new isolates are assigned to anoxygenic phototrophic bacteria of the family *Heliobacteriaceae*, since they possess the unique properties of this taxon: strict anaerobiosis, formation of bacteriochlorophyll *g*, the lack of extensive intracytoplasmic membranes and chlorosomes, an unusual cell wall structure, and phylogenetic relatedness to the low G+C gram-positive eubacteria.

By their phenotypic properties, our new heliobacteria most closely resemble *Heliobacterium gestii* and *Heliobacillus mobilis* (Table 2). However, they differ from these species by the lower temperature optima of growth and the ability to oxidize sulfide in the light. A morphological feature distinguishing our isolates from *Heliobacterium gestii* is the type of flagellation (Table 2). Our isolates also differ from each other by certain phenotypic properties. Cells of strain BR4 are rod-shaped and form endospores, whereas cells of strain BG29 are nonsporeforming spirilla. They also show some differences in the range of organic compounds supporting their photoheterotrophic growth (Table 2). Strains BR4 and BG29 have a similar fatty acid composition, but substantially differ in this aspect from a phenotypically related species *Heliobacterium gestii* (Table 1). The new isolates also showed significant differences in the spectra of total cell proteins (43% similarity). The similarity level of total DNA points to the species-level relationship (more than 25%) between the strains under study and the representatives of the genera *Heliobacterium–Heliobacillus*: *Heliobacterium gestii* and *Heliobacillus mobilis*. At the same time, the DNA–DNA homologies between the new strains of heliobacteria (25%) also allow them to be distinguished within the species level.

The similarity level of the 16S rDNA gene sequences in currently known heliobacteria provides support for the existence of three distinctly isolated genera within the family *Heliobacteriaceae*: *Heliobacterium*, *Heliophilum*, and *Heliorestis* (Fig. 6). Within this group, our new strains fall into cluster 1, including the known species of the genus *Heliobacterium* and *Heliobacillus mobilis*, showing almost the same similarity level of the 16S rRNA gene sequences between each other and the rest of the members of the cluster (94.3–98.0%). It is obvious that in the future, the species *Heliobacillus mobilis* should be placed in the genus *Heliobacterium*, since it appears to be in the cluster that includes only the species of the genus *Heliobacterium*. This suggestion is in line with the phenotypic analysis data (Table 2). The similarities of the 16S rRNA gene sequences were significantly lower (90.5–92.8%), with the members of the other two clusters comprised by the species of the genera *Heliophilum* and *Heliorestis*.

The phenotypic, chemo-, and genosystematic characteristics of the new strains of heliobacteria allow us

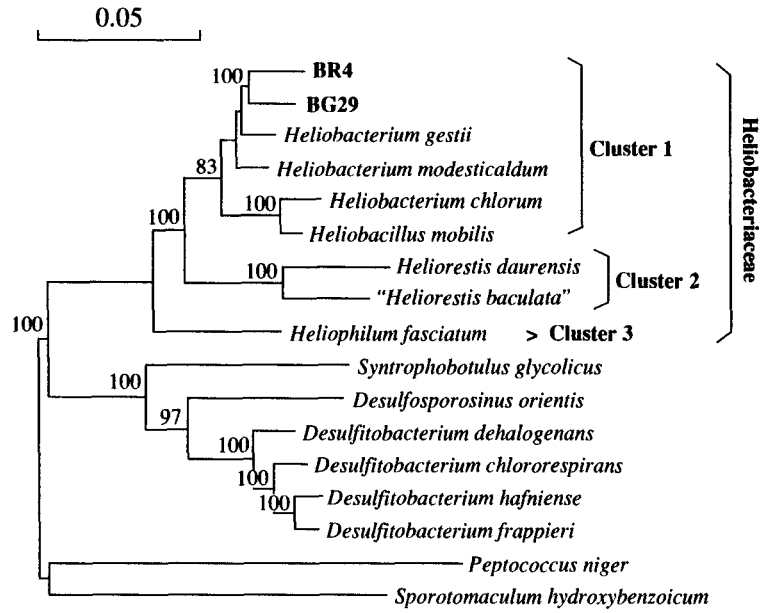

Fig. 7. Phylogenetic position of strain BR4 (*Heliobacterium sulfidophilum*) and strain BG29 (*Heliobacterium undosum*) among the members of the family *Heliobacteriaceae* and other related genera determined by the comparison of their 16S rDNA gene sequences. Pairwise evolutionary distances were computed from five nucleotide changes per 100 nucleotides. Figures show the significance of the branching constructed by the neighbor-joining method with bootstrap analysis of 100 alternative trees. The significance values under 95% are not shown. Bar, evolutionary distance of 0.05.

to classify them as the new species of the genus *Heliobacterium*. We suggest assigning the name *Heliobacterium sulfidophilum* to strain BR4 and *Heliobacterium undosum* to strain BG29.

Description of *Heliobacterium sulfidophilum* sp. nov. *Sulfidophilum* (sul.fi.do'phi.lum. Gr. adj. *philum*, liking; M. L. adj. *sulfidophilum*, liking sulfide).

Cells are rods measuring 0.6–1 by 4–7 μm, motile by peritrichous flagella. Reproduction occurs by cross-septation. Stains gram-negatively, but lacks an outer membrane. Intracytoplasmic membrane or chlorosomes absent. Phototrophic: contains bacteriochlorophyll *g* and carotenoid, by spectral characteristics similar to neurosporene. Absorption maxima of intact cells at 375, 412, 575, 670, and 788 nm. Produces endospores, but loses this ability upon long cultivation. Obligately anaerobic. Grows photoheterotrophically on pyruvate, acetate, malate, lactate, yeast extract, butyrate (+CO_2), and casein hydrolysate as carbon sources, or chemotrophically in the dark at the expense of pyruvate fermentation. Biotin is required as a growth factor. Tolerates up to 2 mM sulfide at pH 7.4, oxidizing it to elemental sulfur when grown photoheterotrophically. Unable to grow photoautotrophically. Reduces elemental sulfur. Optimum growth temperature 32°C, pH range for growth 6.5–9.O with the optimum at 7–8. Tolerates up to 0.5% NaCl, but NaCl is not required.

Habitat: alkaline sulfidic hot springs.

G+C content of DNA 51.3 mol % (T_m).

Type strain BR4^T isolated from a cyanobacterial mat of a sulfidic hot spring near the Bol'shaya River in Buryatia. Deposited in the Collection of Unique Cultures of the Institute of Microbiology, Russian Academy of Sciences (UNIQEM) as No 113.

Description of *Heliobacterium undosum* sp. nov. *Undosum* (un.do'sum. L. adj. *undosum*, curving; M. L. adj. *undosum*, curving).

Cells variable from short rods to slightly twisted spirilla. Cell size varies in the range of 0.8–1.2 by 7–20 μm. Motile by peritrichous flagella. Multiplies by cross-septation. Non-spore-forming. Stains gram-negatively, but lacks an outer membrane. Intracytoplasmic membrane absent. Phototrophic: contains bacteriochlorophyll *g* and carotenoid, by spectral characteristics similar to neurosporene. Absorption maxima of intact cells at 370, 412, 576, 671, 720(S), and 790 nm. Obligately anaerobic. Grows photoheterotrophically on pyruvate, acetate, lactate, propionate, yeast extract, and casein hydrolysate as carbon sources, or chemotrophically in the dark at the expense of pyruvate fermentation. Biotin is required as a growth factor. Tolerates up to 2 mM sulfide at pH 7.5, oxidizing it to elemental sulfur when grown photoheterotrophically. Unable to grow photoautotrophically. Optimum growth temperature 31–36°C, pH range for growth 6.5–9.0 with the optimum at 7–8. Tolerates up to 0.2% NaCl, but NaCl not required.

Habitat: alkaline sulfidic hot springs.

G+C content of DNA 57.2–57.7 mol % (T_m).

Type strain BG29^T isolated from the cyanobacterial mat of the Garginskii sulfidic hot spring located in the Barguzinskaya valley in Buryatia. Deposited in the DSMZ as DSM 13378^T.

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REFERENCES

1. Madigan, M.T. and Ormerod, J.G., Taxonomy, Physiology, and Ecology of Heliobacteria, *Anoxygenic Photosynthetic Bacteria*, Blankenship, R.E., Madigan, M.T., and Bauer, C.E., Eds., Dordrecht: Kluwer, 1995, pp. 17–30.
2. Woese, C.R., Debrunner-Vossbrinck, B.A., Oyaizu, H., Stackebrandt, E., and Ludwig, W., Gram-Positive Bacteria: Possible Photosynthetic Ancestry, *Science*, 1985, vol. 229, pp. 762–765.
3. Starynin, D.A. and Gorlenko, V.M., Sulfide-oxidizing Spore-forming Heliobacteria Isolated from a Hot Sulfide Spring, *Mikrobiologiya*, 1993, vol. 62, no. 3, pp. 556–563.
4. Starynin, D.A., Lysenko, A.M., and Gorlenko, V.M., Taxonomic Position of Heliobacteria Studied by DNA–DNA Hybridization, *Abstracts of the VIII Int. Symp. on Phototrophic Prokaryotes (September 10–15, 1994, Urbino, Italy)*, 1994, p. 97.
5. Kimble, L.K., Mandelco, L., Woese, C.R., and Madigan, M.T., *Heliobacterium modesticaldum*, sp. nov., a Thermophilic Heliobacterium of Hot Springs and Volcanic Soils, *Arch. Microbiol.*, 1995, vol. 163, pp. 259–267.
6. Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I., Achenbach, L.A., and Madigan, M.T., *Heliorestis daurensis*, gen. nov., sp. nov., an Alkaliphilic Rod-to-Coiled-shaped Phototrophic Heliobacterium from a Siberian Soda Lake, *Arch. Microbiol.*, 1999, vol. 172, pp. 167–174.
7. Bryantseva, I.A., Gorlenko, V.M., Kompantseva, E.I., Tourova, T.P., Kuznetsov, B.B., and Osipov, G.A., Alkaliphilic Heliobacterium *Heliorestis baculata* sp. nov. and Emended Description of the Genus *Heliorestis, Arch. Microbiol.* (in press).
8. Pfennig, N. and Lippert, K.D., *Uber das Vitamin* B_{12}-Bidürfnis phototropher Schwefelbakterien, *Arch. Mikrobiol.*, 1966, vol. 55, pp. 245–256.
9. Reznikov, A.A., Mulikovskaya, E.P., and Sokolov, I.Yu., *Metody analiza prirodnykh vod* (Methods for the Analysis of Natural Waters), Moscow: Nedra, 1970, p. 118.
10. Trüper, H.G. and Schlegel, H.G., Sulphur Metabolism in *Thiorhodaceae*: 1. Quantitative Measurements of Growing Cells of *Chromatium okenii, Antonie van Leeuwenhoek*, 1964, vol. 30, pp. 225–238.
11. Laemmli, U.K., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature*, 1970, vol. 227, pp. 680–685.
12. Dice, L.R., Measurement of the Amount of Ecologic Association between Species, *Ecology*, 1945, vol. 26, pp. 297–302.
13. Marmur, J.A., Procedure for the Isolation of Deoxyribonucleic Acid from Microorganisms, *J. Mol. Biol.*, 1961, vol. 3, pp. 208–218.
14. Owen, R.J., Hill, L.R., and Lapage, S.P., Determination of DNA Base Composition from Melting Profiles in Dilute Buffers, *Biopolymers*, 1961, vol. 7, pp. 503–516.
15. De Lay, J., Cattoir, H., and Reynaerts, A., The Quantitative Measurement of DNA–DNA Hybridization from Renaturation Rates, *Eur. J. Biochem.*, 1970, vol. 12, pp. 133–142.
16. Zhilina, T.N., Tourova, T.P., Kuznetsov, B.B., Kostrikina, N.A., and Lysenko, A.M., *Orenia sivashensis* sp. nov., a New Moderately Halophilic Anaerobic Bacterium from Lake Sivash Lagoons, *Mikrobiologiya*, 1999, vol. 68, no. 4, pp. 519–527.
17. Van de Peer, Y. and De Wachter, R., TREECON for Windows: A Software Package for the Construction and Drawing of Evolutionary Trees for the Microsoft Windows Environment, *Comput. Appl. Biosci.*, 1994, vol. 10, pp. 569–570.
18. Taikaichi, S., Inoue, K., Akaike, M., Kobayashi, M., Oh-Oka, H., and Madigan, M.T., The Major Carotenoid in All Species of Heliobacteria is the C_{30} Carotenoid 4,4'-Diaponeurosporene, Not Neurosporene, *Arch. Microbiol.*, 1997, vol. 168, pp. 277–281.
19. Beer-Romero, P., Favinger, J.L., and Gest, H., Distinctive Properties of Bacilliform Photosynthetic Heliobacteria, *FEMS Microbiol. Lett.*, 1988, vol. 49, pp. 451–454.
20. Aase, B., Jantzen, E., Bryn, K., and Ormerod, J., Lipids of Heliobacteria Are Characterised by a High Proportion of Monoenoic Fatty Acids with Variable Double Bond Positions, *Photosynthesis Res.*, 1994, vol. 41, pp. 67–74.