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EXPERIMENTAL ARTICLES

Geoalkalibacter ferrihydriticus gen. nov. sp. nov., the First Alkaliphilic Representative of the Family *Geobacteraceae*, Isolated from a Soda Lake

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Abstract—Investigation of iron reduction in bottom sediments of alkaline soda lakes resulted in the isolation of a new obligately anaerobic iron-reducing bacterium, strain Z-0531, from Lake Khadyn (Tuva, Russia) sediment samples. The cells of strain Z-0531 are short (1.0–1.5 by 0.3–0.5 μm), motile, non-spore-forming, gram-negative rods. The isolate is an obligate alkaliphile, developing in the pH range of 7.8–10.0, with an optimum at pH 8.6. It does not require NaCl but grows at NaCl concentrations of 0–50 g/l. It can oxidize acetate with such electron acceptors as amorphous Fe(III) hydroxide (AFH), EDTA-Fe(III), anthraquinone-2,6-disulfonate (quinone), Mn(IV), and S^0. On medium with EDTA-Fe(III), the isolate can oxidize, apart from acetate, ethanol, pyruvate, oxalate, arginine, tartrate, lactate, propionate, and serine. H_2 is not utilized. The reduced products formed during growth with AFH are siderite or magnetite, depending on the growth conditions. The isolate is incapable of fermenting sugars, peptides, and amino acids. Yeast extract or vitamins are required as growth factors. The organism is capable of dinitrogen fixation and harbors the *nifH* gene. The DNA G+C content is 55.3 mol %. 16S rRNA analysis places strain Z-0531 into the family *Geobacteraceae*. Its closest relative (93% similarity) is *Desulfuromonas palmitatis*. Based on phenotypic distinctions and phylogenetic position, it is proposed that this strain be assigned to the new genus and species *Geoalkalibacter ferrihydriticus* gen. nov., sp. nov. (Z-0531^T-DSMZ-17813-VKMB-2401).

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Key words: Iron reduction, soda lakes, alkaliphiles, anaerobes, dinitrogen fixation, *Geobacteraceae*.

The discovery in the 1980s, of the ability of microorganisms to reduce ferric iron and the isolation of bacteria gaining energy in the course of this reaction impelled microbiologists to active studies of the iron reduction process [1–3]. Over the recent period, a large number of bacteria capable of dissimilatory iron reduction and a much smaller number of archaea have been isolated [4–6]. In addition, it has been shown that many microorganisms from other physiological groups, such as methanogens and sulfate reducers, are also capable of iron reduction [7–9]. The proximity of the phenotypic and phylogenetic characteristics of the bacteria belonging to eight genera (*Geobacter, Desulfuromonas, Desulfuromusa, Pelobacter, Geothermobacter*, *Geopsychrobacter*, *Malonomonas*, and *Trichlorobacter*) allowed the new family *Geobacteraceae* to be described, whose diagnostic characteristic is the capacity to reduce sulfur and/or iron [10, 11]. In addition to subterranean ecosystems, iron reducers are widespread in soils and marine and freshwater reservoirs [12]. Apart from mesophiles, this group includes thermophilic [6] and psychrophilic [13, 14] representatives.

Most iron reducers are mesophilic and neutrophilic organisms, adapted to the conditions prevailing on the Earth's surface [4]. However, acidophilic iron reducers are also known; they have been isolated primarily from drainage waters of coal and sulfide ore mines [15–17]. It can be seen from our brief review that the capacity for iron reduction is widespread in the microbial world and that the habitats of the currently known iron reducers include virtually all possible ecological niches.

However, under alkaline conditions, exemplified by soda lakes, only one representative of dissimilatory iron reducers has been found, namely *Alkaliphilus metalliredigens*, isolated from an alkaline pond formed at a site of boron extraction in the United States [18]. In addition, the ability to reduce ferric iron in an NTA–Fe(III) complex has been revealed for cell suspensions

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Contents of chloride and carbonates in the soda lakes studied

Lake	NaCl, g/l	Na_2CO_3 g/l	$NaHCO_3$ g/l	pH
Magadi	60	68.0	38.0	10.0
Khilganta	20	0.5	2.0	9.5
Nizhnee Beloe	1.2	3.08	2.2	9.6
Khadyn	1.0	3.0	10.0	9.5

of *Bacillus arsenicoselenatis* [19]. The thermoalkaliphilic bacterium *Anaerobranca californiensis* should also be mentioned, which reduces iron in the presence of peptides [20]. The possibility of reduction of amorphous and weakly crystalline iron oxides in alkaline environments has been subject to doubt [18] due to the low mobility of Fe(III) under these conditions.

In the present work, we provide a taxonomic description of the first alkaliphilic representative of the family *Geobacteracea*, isolated from a bottom sediment sample from the weakly mineralized soda lake Khadyn (Tuva, Russian Federation) and capable of reducing various ferric iron compounds, including amorphous Fe(III) hydroxide (AFH).

MATERIALS AND METHODS

Isolation source. This work used samples of anoxic bottom sediments of four soda lakes: Lake Magadi (Kenya; pH 10.0; mineralization, 250 g/l), Lake Khilganta (Buryatia, Russian Federation; pH 9.5; mineralization, 60 g/l), Lake Nizhnee Beloe (Buryatiya, Russian Federation; pH 9.7; mineralization, 10 g/l), and Khadyn (Tuva, Russian Federation; pH 9.5; mineralization, 17 g/l).

Cultivation conditions. Enrichment cultures were obtained by introducing 1 g of sediment into 100 ml of medium. Sodium acetate (2 g/l) was employed as the electron donor, and AFH (90 mM), prepared by titration of a $FeCl_3$ solution with 10% NaCl, was used as the electron acceptor. The medium also contained (g/l) KH_2PO_4, 02; $MgCl_2$, 0.1; NH_4Cl, 0.5; KCl, 0.2; yeast extract, 0.1; sodium acetate, 2, and Wolin trace element solution, 1 ml/l. The concentrations of NaCl, Na_2CO_3, and $NaHCO_3$ were different for the enrichments raised from samples taken from different lakes and corresponded to the in situ concentrations (see the table).

Further isolation of iron-reducing microorganisms from enrichment cultures was carried out on medium in which AFH was substituted for by EDTA–Fe(III) (20 mM). 100 ml of a 0.4 M Na_2–EDTA solution was mixed with 100 ml of 0.4 M $FeCl_3 \cdot 6H_2O$ and titrated with 10% NaOH to pH 7.5–8.0. The solution was filter-sterilized and added to autoclaved medium in the amount of 100 ml/l. Incubation was performed in the dark at 37°C for two weeks.

Isolation of the pure culture, designated strain Z-0531, was performed by multiple repeated culture transfers from serial dilutions, since the microorganism did not produce colonies on agar medium.

The isolate was cultured on an optimized medium of the following composition (g/l): KH_2PO_4, 0.2; $MgCl_2$, 0.1; NH_4Cl, 0.5; KCl, 0.2; NaCl, 1.0; Na_2CO_3, 3.0; $NaHCO_3$, 10.0; yeast extract, 0.1, acetate, 1; trace element solution [21], 1 ml/l; pH 9.5. Na_2S was not added because it binds with Fe(III).

Since the organism did not form colonies on agarized media, the pure culture designated Z-0531^T was isolated by repeated sequential 10-fold dilutions.

Physiological characteristics. When testing the ability of the isolate to utilize various electron donors, sodium acetate in the medium was replaced with organic acids, sugars, or peptides in a concentration of 3 g/l, or alcohols (5 ml/l) or molecular hydrogen (100% in the gas phase). Acceptors were added from stock solutions to sterile medium to obtain the following concentrations (mM): $Na_2S_2O_4$, 1; Na_2SO_3, 2 and 10; $NaNO_2$, 2 and 10; $NaNO_3$, 20; $Na_2S_2O_3 \cdot 5H_2O$, 20; Na_2SO_4, 20; Fe(III) citrate, 5; anthraquinone-2,6-disulfonate, 20; Mn(IV), 25, in the form of artificially synthesized MnO_2; S^0, 2% (wt/vol).

In the experiments on the pH dependence of growth, sodium carbonate in the medium was replaced with a tenfold lesser sodium bicarbonate concentration (the molar concentration of sodium was equalized with sodium chloride), and the required pH values were obtained by titration with 10% HCl or 10% NaOH. The temperature dependence of growth was studied in the 18– 40°C range.

The dependence of growth on sodium carbonates was studied by replacing them with an equimolar amount of Na_2SO_4 (the pH was maintained at 9.2 with a Tris buffer or at pH 9.7 with 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (50 mM)). The requirement for NaCl was studied by replacing it with an equimolar amount of a sodium carbonate–sodium bicarbonate mixture; all other chloride salts in the medium were replaced with sulfates. In both cases, pyruvate and S^0 were employed as the electron donor and acceptor because the procedure for obtaining a solution of the EDTA–Fe(III) complex inevitably results in the appearance of NaCl in the solution (see above).

Nitrogenase activity. The capacity to fix dinitrogen was tested on medium supplemented with pyruvate and S^0 and devoid of nitrogen sources other than N_2; the medium was amended with yeast extract (0.1 g/l) or Wolin vitamin solution (10 ml/l) as growth factors. The presence of nitrogenase activity was verified by the acetylene method [22]. The strains under study were grown anaerobically in Hungate tubes with 10 ml of medium and a 6.5-ml gas phase. After three consecutive culture transfers, acetylene was introduced into the gas phase (10%), and, after 24 h of incubation in the dark at

35°C, the ethylene formed was determined on a model GKh 3700 gas chromatograph.

The cytochrome composition was determined from difference spectra of dipyridyl-treated membrane preparations (dithionite-reduced versus H_2O_2-oxidized). The spectra were recorded at ambient temperature in 1-cm cuvettes on a Cary 100 Bio (Varian, United States) spectrophotometer in the 300–600 nm range. To obtain the membrane preparations, biomass sedimented by centrifugation was treated ultrasonically for 15 s and resuspended in phosphate buffer.

Analytical methods. Acetate and ethanol oxidation were determined by HPLC on a Staier (Russia) chromatograph. Fe(III) reduction was determined colorimetrically in a reaction with rhodanide after dissolution of the precipitate in fuming HCl. Dissolved hydrogen sulfide was determined colorimetrically from the formation of methylene blue. Optimal conditions were determined by the decoloration rate of the EDTA–Fe(III) complex, which correlated with the decrease in the Fe(III) content in the solution due to bacterial growth. The reduced solid phase was investigated by Mõsbauer spectroscopy.

Morphology. Living cells were examined under a ZETOPAN phase-contrast microscope (Austria). Thin sections and whole cells stained with phosphotungstic acid to reveal flagella were examined under a JEM-100C electron microscope (Jeol, Japan).

The G+C content of DNA was determined as described earlier [21], using *Escherichia coli* LB DNA as a standard.

Isolation of DNA and amplification and sequencing of the genes studied. Isolation and purification of DNA were performed according to the procedures described earlier [23].

Amplification and sequencing of 16S rRNA genes employed primers universal for bacteria [24], and amplification of *nifH* genes used primer systems that were developed earlier [25].

Sequencing of the amplification products was performed by the Sanger method on an ABI 3730 automatic sequencer (Applied Biosystems, United States) with the use of a Big Dye Terminator v.3.1 kit according to the manufacturer's recommendations.

Phylogenetic analysis of gene sequences. The sequences were edited using the BioEdit software package (http://jwbrown.mbio.nscu.edu/BioEdit/bioedit.html). Primary comparative analysis of the newly determined sequences with sequences for the GenBank database was performed with the use of the NCBI BLAST software (http://www.ncbi.nlm.nih.gov/blast). Further comparative analysis employed *rrs* and *nifH* gene sequences available from GenBank. Nucleotide sequences and deduced amino acid sequences were aligned with the corresponding closely related sequences with the use of the CLUSTALW v 1.75 software package. Phylogenetic trees were constructed using the algorithms implemented in the TREECONW (http://bioc-www.uia.ac.be/u/yvdp/treeconw.html) and PHYLIP (http://evolution.genetics.washington.edu/phylip.html) software packages. The statistical significance of the branching order was determined by bootstrap analysis of 1000 alternative trees.

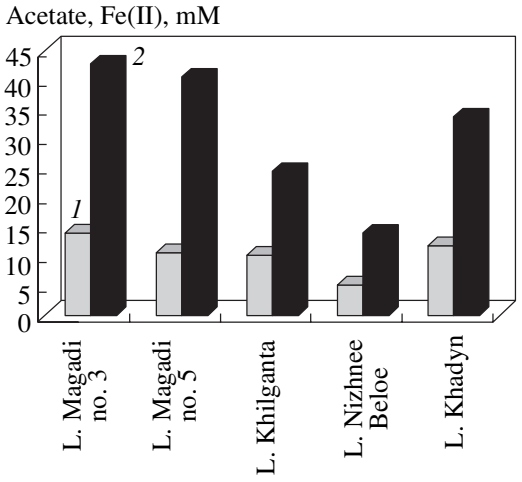

Fig. 1. Oxidation of acetate (*1*) and reduction of iron (*2*) in enrichment cultures from bottom sediments of soda lakes.

Deposition of nucleotide sequences. The nucleotide sequences of the 16S rRNA and *nifH* gene fragments have been deposited in GenBank under accession numbers DQ309326 and DQ660332.

RESULTS

The process of AFH reduction, accompanied by sediment blackening and acetate oxidation, started in all of the samples investigated 4–7 days after inoculation. The processes of acetate oxidation and iron reduction were most active in sample no. 3 from highly mineralized Lake Magadi and in a sample from weakly mineralized Lake Khadyn (Fig. 1). After five consecutive transfers of the culture derived form the sample from Lake Khadyn on medium with AFH, we obtained a stable enrichment that reduced iron and contained cells of several morphotypes, mainly rods, which were spore-forming or non-spore-forming and varied in length and width. To alleviate the isolation procedure, the enrichment was further grown on a medium that contained EDTA–Fe(III) instead of AFH. In addition, we decreased the concentration of yeast extract to 0.05 g, which allowed us to eliminate peptolytic microorganisms. As a result, after several transfers, the culture contained only one dominant type of cells, which were weakly motile short rods. The attempts to obtain colonies of this bacterium on agarized medium failed. By using the serial dilutions method, we obtained, from the 10^{-6} dilution, a pure bacterial culture, which we designated strain Z-0531. The transfer of strain Z-0531 to medium with AFH confirmed the ability of the culture to reduce this compound.

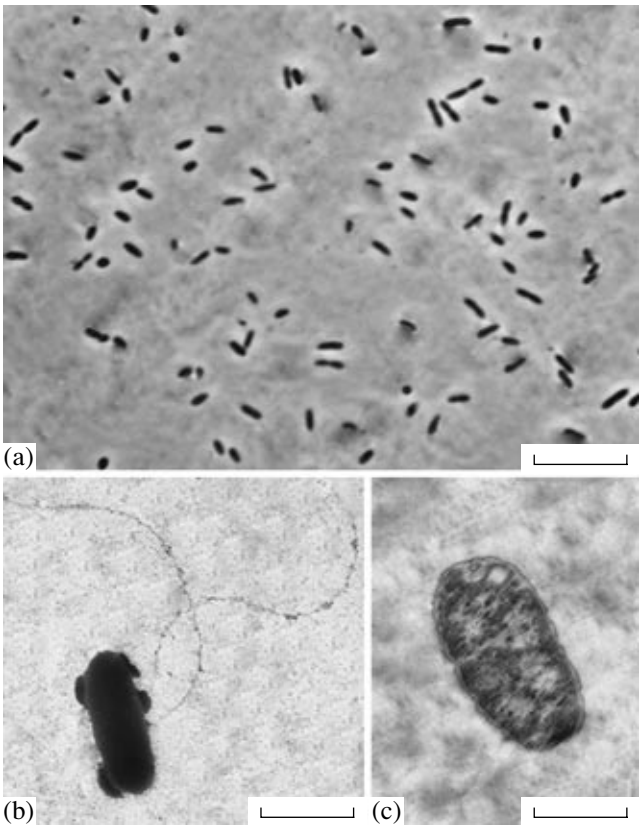

Fig. 2. Morphology of cells of strain Z-0531 grown on medium with acetate and EDTA–Fe(III): (a) Light microscopy; bar, 10 μm; (b) electron microscopy of cells with lateral flagella; bar, 1.0 μm; and (c) electron microscopy of a longitudinal thin section of a cell with a gram-negative cell wall type; bar, 0.5 μm.

Morphological characteristics. The morphology of strain Z-0531 somewhat varied in dependence on the growth substrate. Acetate-grown cells were short weakly motile rods measuring 1.0–1.5 × 0.3–0.5 μm (Fig. 2a), whereas, during growth on pyruvate, some of the cells were as long as 5 μm and exhibited higher motility. Cells occurred singly or in pairs. The motility was due to one to three lateral flagella (Fig. 2b). The cells exhibited high adsorption capacity toward glass and mineral particles.

Growth characteristics. Strain Z-0531 was an obligate alkaliphile with a growth pH range of 7.6–10.0 and a pH optimum of 8.6 (Fig. 3). It was a mesophile growing at 18–39°C, with an optimum at 35°C. The strain was obligately anaerobic; it could not grow on aerobically prepared medium or in the presence of 2% O_2. Growth was, however, possible in the absence of reducing agents. At the same time, a decrease in the redox potential due to the addition of cysteine (0.3 g/l) to the medium resulted in an increase in the growth rate and the rate of AFH reduction (Fig. 4). The organism did not require NaCl; however, it could grow at NaCl concentrations of up to 50 g/l; i.e., it was halotolerant

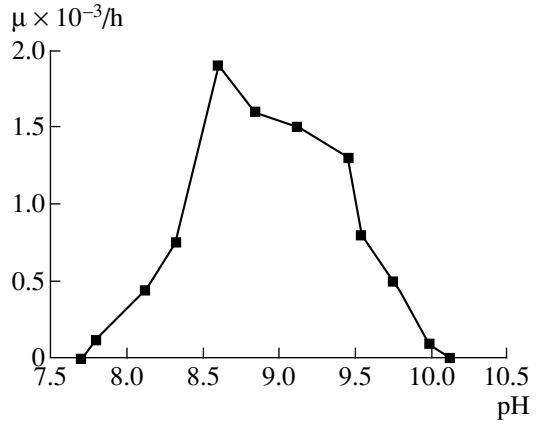

Fig. 3. Dependence of the growth rate of strain Z-0531 on pH.

(Fig. 5). Carbonates were also not indispensable and could be replaced with Tris or CAPS buffer.

Strain Z-0531 turned out to be incapable of fermentation; it did not use arabinose, galactose, glucose, xylose, lactose, maltose, mannose, sucrose, sorbose, rhamnose, ribose, trehalose, fructose, fucose, peptone, yeast extract, meat extract, tryptone, Casamino acids, or acetyl-D-glucosamine. When EDTA–Fe(III) was provided as an electron acceptor, the strain, besides acetate, could utilize pyruvate, oxalate, ethanol, tryptone, tartrate, arginine, serine, propionate, and lactate (the substrates are listed in the order of decreasing growth rate). With the above donors, the growth was shown to be stable over three culture transfers. The strain did not oxidize betaine, butyrate, glycerol, yeast extract, dulcitol, inositol, malonate, mannitol, sorbitol, succinate, peptone, formate, methanol, propanol, or H_2.

Ethanol oxidation was incomplete and proceeded until acetate formation. The ratio of acetate oxidized to iron reduced to cells produced was 9.3 mM : 41.3 mM : 8.5×10^7 cells/ml (Fig. 6). On medium with AFH, the reduced mineral products were siderite or magnetite.

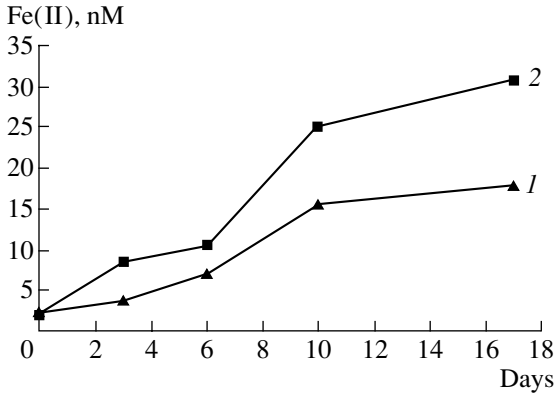

Fig. 4. Effect of the addition of 0.3 g/l of cysteine on the rate of AFH reduction by strain Z-0531: (*1*) medium without cysteine; (*2*) medium with cysteine.

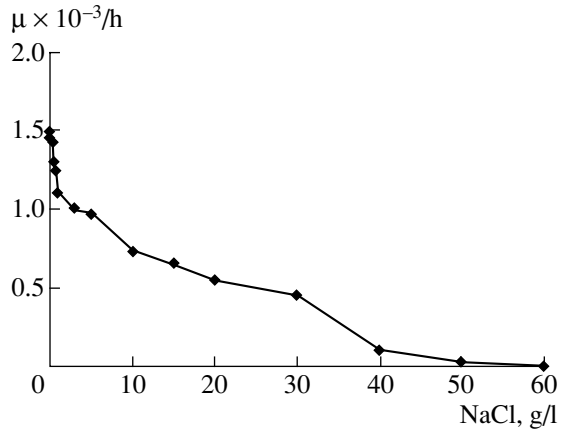

Fig. 5. Dependence of the growth rate of strain Z-0531 on NaCl concentration.

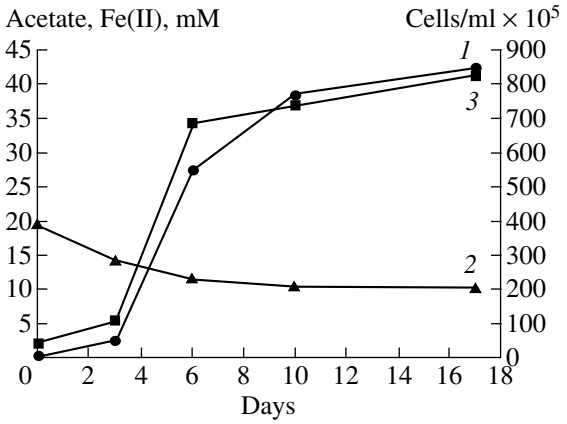

Fig. 6. Growth of strain Z-0531 on medium with acetate and AFH: (*1*) cell number; (*2*) acetate oxidation; (*3*) AFH reduction.

During growth with acetate as an electron donor, strain Z-0531 exhibited stable capacity for the reduction of quinone, Mn(IV), and S^0. Quinone reduction was recorded by the change in the medium coloration from pale yellow to dark brown (comparison with an abiogenic control was made). The reduction of MnO_2 was accompanied by a change in the sediment color from dark brown to white due to the formation of rhodochrosite (comparison with abiogenic control was made). Reduction of S^0 resulted in the formation of up to 20.5 mM H_2S. The addition of 0.1 g/l of quinone to AFH-containing medium increased the rate of AFH reduction 2- to 3-fold (Fig. 7). Fe(III) citrate, nitrate, nitrite, sulfate, sulfite, thiosulfate, and fumarate were not used by strain Z-0531 as electron acceptors.

In the membranes of strain Z-0531 cells, we identified a cytochrome *c* typical of *Geobacteraceae* representatives (the absorption maxima were at 552, 523, and 420 nm, Fig. 8).

Strain Z-0531 growth was not affected by 100 mg/l vancomycin or kanamycin and was inhibited by chloramphenicol, streptomycin, penicillin, ampicillin, novobiocin, bacitracin, and rifampicin.

The G+C content of strain Z-0531 DNA was 55.3 ± 1 mol %.

Capacity for dinitrogen fixation. A characteristic feature of the family *Geobacteraceae* representatives is the presence of the *nifD* gene [11], which indicates the potential ability of these bacteria to fix dinitrogen. In order to check the capacity of strain Z-0531 for dinitrogen fixation, it was inoculated into a medium devoid of nitrogen sources (except for N_2 in the gas phase). The bacterium was shown to be able to grow in this medium after three consecutive culture transfers. The cells of strain Z-0531 could reduce acetylene to ethylene during growth on medium containing pyruvate, S^0, and yeast extract or Wolin vitamin solution (10 ml/l). S^0 reduction was more efficient in the latter variant of the medium. The nitrogenase activity was not high (1.08 mM C_2H_2/h per 5.5×10^8 cells of the culture). However, the presence of nitrogenase and the growth on medium free of bound nitrogen demonstrate the capacity of strain Z-0531 for dinitrogen fixation.

Analysis of 16S rRNA genes. Almost complete sequencing of the strain Z-0531 16S rRNA gene was carried out (1504 nucleotides corresponding to *Escherichia coli* positions 11–1496). According to the results of preliminary screening in the GenBank database, the nucleotide sequence of the strain Z-0531 16S rRNA gene was closest to the analogous sequences of representatives of the family *Geobacteraceae* within the class *Deltaproteobacteria.* In the phylogenetic tree constructed (Fig. 9a), the strain Z-0531 16S rRNA gene formed a separate branch within the cluster that combined representatives of the family *Geobacteraceae.* The similarity levels between the 16S rRNA gene of strain Z-0531 and the described representatives of this family did not exceed 93% and were approximately the

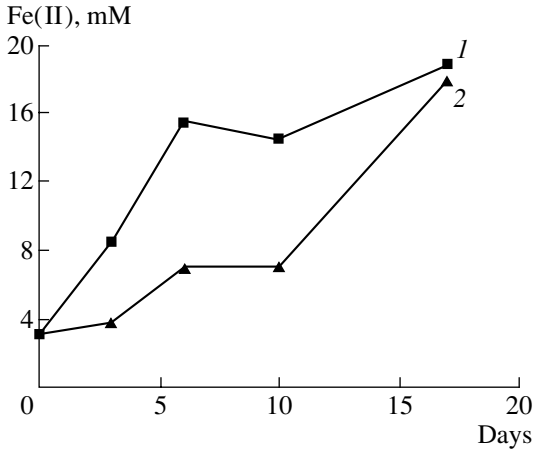

Fig. 7. Effect of the addition of 0.1 g/l of quinone on the rate of AFH reduction by strain Z-0531: (*1*) medium without quinone; (*2*) medium with quinone.

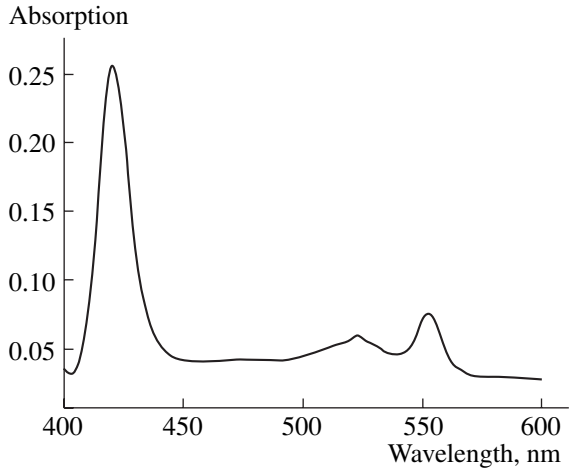

Fig. 8. Difference spectrum of strain Z-0531 cytochrome *c*.

same with representatives of different species, comprising 89.9–92.8% with *Desulfuromonas* species, 87.1–89.7 with *Geobacter* species, 89.6–90.4% with *Desulfuromusa* species, 85.9–90.2% with species of *Pelobacter*, and 85.9–90.2% with the single species of the genera *Geothermobacter, Geopsychrobacter, Malonomonas*, and *Trichlorobacter.*

Analysis of the *nifH* gene. By using an earlier developed [23] oligonucleotide primer system, we revealed in strain Z-0531 a *nifH* gene fragment (about 450 nucleotides) and sequenced it. According to preliminary BLAST analysis in the GenBank database, the gene fragment was most similar to analogous genes of deltaproteobacteria of the genera *Desulfuromonas, Geobacter*, and *Pelobacter.*

The alignment of the deduced amino acid sequence corresponding to the *nifH* gene fragment of strain Z-0531 with analogous sequences of dinitrogen-fixing proteobacteria, available in GenBank, allowed us to compare 150 positions of amino acid residues.

In the phylogenetic tree constructed (Fig. 9b), strain Z-0531 clustered (with a bootstrap support level of 78%) with representatives of the genera *Desulfuromonas, Geobacter*, and *Pelobacter.* The identity level of its amino acid sequence with sequences belonging to representatives of the above genera varied from 85.1 to 91.5% (the table). These values were similar to those characterizing the relationships between analogous sequences of representatives of *Desulfuromonas, Geobacter*, and *Pelobacter* (89.0–95.1%) and were considerably higher than the similarity recorded with other deltaproteobacteria (63–71%). This indicates the common evolutionary origin of nitrogenase genes in strain Z-0531 and representatives of the genera *Desulfuromonas, Geobacter*, and *Pelobacter.*

DISCUSSION

Our investigations of the processes of dissimilatory iron reduction with acetate as an electron donor and AFH as an electron acceptor were carried out with samples of bottom sediments from four soda lakes differing in their alkalinity and NaCl content. Omission of sulfate from the medium composition provided for the absence of sulfate-reducing competitors. In all of the samples studied, reduction of AFH occurred, accompanied by the acquisition of black color by the sediment. The intensity of the process was judged from the rates of acetate oxidation and iron reduction. The results obtained show (1) that the iron reduction process widely occurs in soda lakes, (2) that the intensity of the process is independent of alkalinity and mineralization (acetate oxidation and iron reduction were most intense in sample no. 3 from highly mineralized Lake Magadi, in which sedimentation of halite and trona takes place, and in the weakly mineralized Lake Khadyn), and (3) that alkaliphilic iron reducers are capable of AFH reduction.

Until our work, it was believed [18] that the reduction of insoluble iron compounds is limited by pH, since soluble iron at pH above 9.0 is unavailable; the earlier isolated iron reducers were able to reduce only artificial soluble iron compounds, such as NTA- or EDTA-Fe(III) [18]. Our success in obtaining enrichments with AFH allow us to state with certainty that, even under alkaline conditions, insoluble iron compounds can undergo microbial reduction.

In addition, the active oxidation of acetate in all of the samples studied allows us to assume an important role of alkaliphilic iron reducers at the terminal stage of organic matter decomposition in anaerobic microbial communities, since it is known [26] that the oxidation of acetate, accumulated in the process of organic matter decomposition, is a problem for the anaerobic zone of soda lakes.

The bacterium that we isolated from the bottom sediments of Lake Khadyn is a typical chemoorganotrophic organism, utilizing a rather wide range of organic acids and ethanol as electron donors. The ability of the isolate to oxidize acetate allows it to be placed at the very end of the trophic chain of the alkaliphilic microbial community, in which it plays the role of a secondary anaerobe completing the mineralization of organic matter. At present, it is known that acetate oxidation in the anaerobic zone of soda lakes may be accomplished by alkaliphilic nitrate reducers belonging to the genus *Halomonas* [27]. Acetate can also be oxidized syntrophically by the pair of an acetate-utilizing bacterium and a hydrogenotrophic sulfate reducer of the genus *Desulfonatronum* or *Desulfonatronovibrio*, as has been shown for bottom sediments of Lake Khadyn [28]. The third possible pathway of acetate oxidation under anaerobic conditions is provided by anoxygenic phototrophs [26]. The isolation of an acetate-

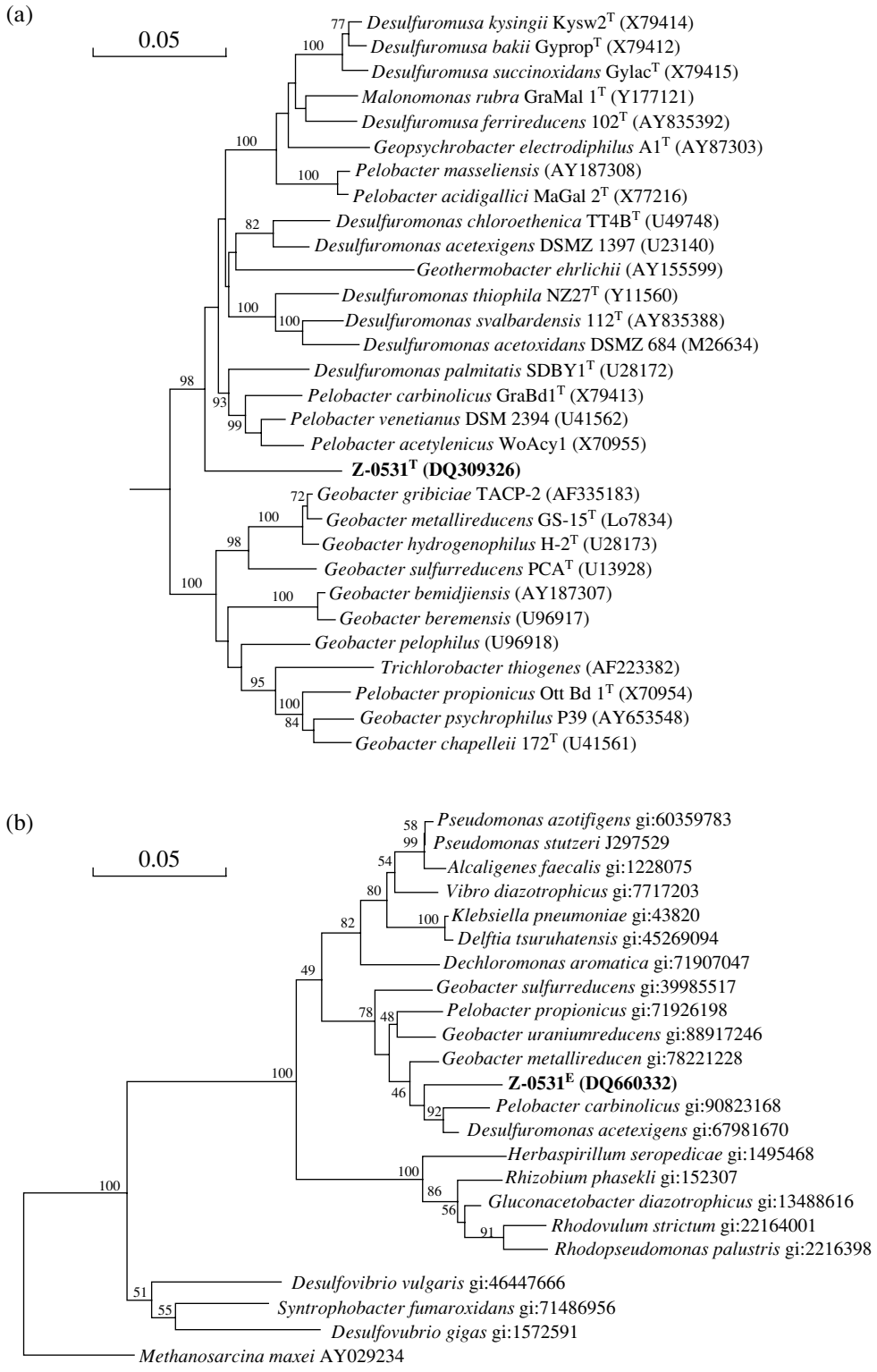

Fig. 9. Position of strain Z-0531 in the phylogenetic trees of the family *Geobacteraceae*, based on comparative analysis of the sequences of (a) 16S rRNA genes and (b) *nifH* genes. Scale bars correspond to 5 substitutions per 100 residues. Numerals show the significance of the branching order as determined by bootstrap analysis (values above 70% were considered significant).

utilizing iron reducer demonstrates the existence of one more pathway of acetate oxidation in soda lakes.

Our isolate turned out to be incapable of lithotrophic growth: it did not use H_2. It should be noted that iron reducers are usually able to utilize

either molecular hydrogen or acetate, and only a few species can use both H_2 and acetate as electron donors [6].

Out of the range of the electron acceptors tested, strain Z-0531 reduced Fe(III) (the Fe(III)–EDTA complex and AFH), quinone, Mn(IV), and S^0. Fe(III) reduction by alkaliphilic bacteria was first shown for *Tindallia magadii* [29], Then, the ability to reduce AFH with peptone was found in *Anoxynatronum sibiricum*. However, in both cases iron reduction was nonspecific, since the cell yield was independent of the presence of Fe(III) [30]. Thus, our isolate Z-0531 is the first alkaliphilic dissimilatory nitrate reducer capable of reduction of insoluble iron compounds as electron acceptors. The reduced minerals formed were siderite and magnetite, as demonstrated by Mõsbauer spectroscopy. The addition of 0.1 g/l of quinone considerably increased the rate of AFH reduction. It has been supposed [8] that quinones play the role of extracellular electron carriers from cells to insoluble iron oxides. This is actually a biologically mediated form of iron reduction, and, to fulfill its function, quinone may be present in a low concentration. Most probably, the stimulatory effect of quinone is related to its ability to form complexes. The rates of growth and AFH reduction were also influenced by the Eh of the medium. The addition of 0.3 g/l of cysteine increased the rate of growth and the amount of AFH reduced (Fig. 5). The stimulatory effect of cysteine can also be explained by its utilization as a sulfur source, since the basal medium was very poor in sulfur compounds.

The capacity to use S^0 and/or Fe(III) as electron acceptors is a characteristic feature of the family *Geobacteraceae*, to which our isolate belongs. The use of sulfur compounds as electron acceptors is urgent in the natural habitats of our isolate: for alkaline soda lakes, the domination of the sulfur cycle is a pronounced feature. Most probably, strain Z-0531 uses polysulfides, whose concentration is maximal precisely under alkaline conditions. This assumption is confirmed by the disappearance of the characteristic yellow coloration of the medium, determined by the presence of polysulfides, in the process of strain Z-0531 growth. During the reduction of S^0, up to 20.5 mM H_2S was accumulated, indicating the high tolerance of our isolate to sulfide.

The bacterium proved to be capable of growing in the presence of up to 50 g/l NaCl. The best growth was observed at 0–3 g/l NaCl (Fig. 6). Thus, the isolate is a halotolerant organism. Its ability to survive salt concentrations tenfold exceeding the optimal one is undoubtedly related to the peculiarities of its natural habitat. The continental climate, with its high summer and low winter temperatures, and the small depth of soda lakes results in their complete or partial drying out in summer and freezing in winter.

According to data of 16S rRNA analysis, strain Z-0531 is a member of the family *Geobacteraceae*, which includes eight genera. The following characteristics are common to *Geobacteraceae* representatives [11]:

(1) the morphology of cells, which are motile or nonmotile rods of varying length with a gram-negative cell wall type;

(2) chemoorganotrophic or chemoautotrophic type of nutrition with the utilization of various iron and/or sulfur compounds as electron acceptors;

(3) capacity for oxidation of acetate and some other organic compounds and, in some cases, molecular hydrogen;

(4) presence of cytochrome *c* and the *nifD* gene (an exception is the genus *Pelobacter*);

(5) neutrophilic growth preferences.

From the above, it can be seen that the family is rather homogeneous phenotypically. Description of new genera is mainly based on data from 16S rRNA gene sequencing and such phenotypic properties as, e.g., phsychrophily (*Geopsychrobacter* [13]). Phenotypic differentiation of species is mainly based on the spectrum of the electron donors utilized.

The presence of the *nifD* gene in all representatives of the family compelled us to search for such genes in strain Z-0531 and to check its capacity for dinitrogen fixation (it is known that the presence of genes responsible for dinitrogen fixation does not unambiguously prove the presence of this capacity). PCR analysis and further sequencing revealed the presence of the *nifH* gene. The amplified *nifH* gene fragment of strain Z-0531 showed 90.8 and 91.5% identity with the most closely related *Desulfuromonas acetoxidans* and *Geobacter metallireducens*; this confirms the affiliation of strain Z-0531 with the family *Geobacteraceae* and demonstrates that it stands apart from the other members. Strain Z-0531 is the third representative of *Geobacteraceae* for which dinitrogen fixation capacity has been demonstrated. Earlier, this capacity was demonstrated in *G. metallireducens* and *G. sulfurreducens* [11].

As mentioned above, the levels of 16S rRNA similarity of strain Z-0531 with members of other *Geobacteraceae* genera are approximately the same and do not exceed 93%; i.e., the distinctions are of the generic level. Based on the phenotypic and genotypic distinctions of strain Z-0531 from other *Geobacteraceae* representatives, we propose that it should be assigned to a new genus and species, *Geoalkalibacter ferrihydriticus* gen. nov., sp. nov.

Description of Geoalkalibacter gen. nov.

Geo.al.ka.li.bac.ter Gr. n. *ge*, earth; N.L. n. *alkali*, from Arabian adjective *al qaliy*, alkaline; N.L. masc. n. *bacter*, from Greek noun *bacterion*, rod; N.L. masc. n. *Geoalkalibacter*, a rod from alkaline earth.

Cells are short, straight or sometimes slightly curved, weakly motile rods. Spores are not formed. The cell wall structure is of the gram-negative type. Obligately alkaliphilic, anaerobic, mesophilic. The type of nutrition is chemoautotrophic. Various compounds of Fe(III) or sulfur are used as electron acceptors. Acetate, some other organic acids, and ethanol are used as electron donors. Cells contain cytochrome *c*. The genus *Geoalkalibacter* belongs to the family *Geobacteraceae* within the class *Deltaproteobacteria.* The type species of the genus is *Geoalkalibacter ferrihydriticus*.

Description of Geoalkalibacter ferrihydriticus sp. nov.

Fer.ri.hy.dri'ti.cus. N.L. masc. adj. *ferrihydriticus*, derived from ferrihydrite, weakly crystalline iron hydroxide, which is reduced by the species.

Cells are short (1.0–1.5 × 0.3–0.5 μm) rods motile by means of one to three lateral flagella. The cell-wall structure is of the gram-negative type. Spores are not formed. Anaerobic, obligately alkaliphilic (growth pH range, 7.8–10.0 with an optimum at 8.6), and mesophilic (growth temperature range, 18– 39°C, with an optimum at 35°C). NaCl is not required, but growth is possible at 0–50 g/l NaCl. Acetate is oxidized with such electron acceptors as amorphous Fe(III) hydroxide, EDTA–Fe(III), anthraquinone-2,6-disulfonate (quinone), Mn(IV), and S^0. Acetate, pyruvate, oxalate, ethanol, tryptone, tartrate, arginine, serine, propionate, and lactate can be used as electron donors on medium with EDTA–Fe(III). H_2 is not used as an electron donor. During growth on amorphous Fe(III) hydroxide, the reduced products are siderite or magnetite. The anabolism requires yeast extract or vitamins. Dinitrogen is fixed. Cells contain cytochrome *c*. The G+C content of DNA is 55.3%.

The habitat is bottom sediments of soda lakes. The type strain was isolated from bottom sediment of weakly mineralized Lake Khadyn (Tuva).

The type strain is Z-0531^T (DSMZ 17813 = VKM B-2401).

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REFERENCES

1. Balashova, V.V. and Zavarzin, G.A., Anaerobic Reduction of Iron Oxide by a Hydrogen-Oxidizing Bacterium, *Mikrobiologiya*, 1979, vol. 48, no. 6, pp. 773–778.
2. Lovley, D.R. and Phillips, E.J.P., Organic Matter Mineralization with the Reduction of Ferric Iron in Anaerobic Sediments, *Appl. Environ. Microbiol.*, 1986, vol. 51, pp. 683–689.
3. Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A., and Goodwin, S., *Geobacter metallireducens* gen. nov., sp. nov., a Microorganism Capable of Coupling the Complete Oxidation of Organic Compounds to the Reduction of Iron and Other Metals, *Arch. Microbiol.*, 1993, vol. 159, pp. 336–344.
4. Straub, K.L., Benz, M., and Schink, B., Iron Metabolism in Anoxic Environments at Near Neutral pH, *FEMS Microbiol. Rev.*, 2001, vol. 34, pp. 181–186.
5. Lovley, D.R., Holmes, D.E., and Nevin, K.P., Dissimilatory Fe(III) and Mn(IV) Reduction, *Adv. Microb. Physiol.*, 2004, vol. 49, pp. 219–286.
6. Slobodkin, A.I., Thermophilic Microbial Metal Reduction, *Mikrobiologiya*, 2005, vol. 74, no. 5, pp. 581–595 [*Microbiology* (Engl. Transl.), vol. 74, no. 5, pp. 501–514].
7. Vargas, M., Kashefi, K., Blunt-Harris, E.L., and Lovley, D.R., Microbiological Evidence for Fe(III) Reduction on Early Earth, *Nature*, 1998, vol. 395, pp. 65–67.
8. Bond, D.R. and Lovley, D.R., Reduction of Fe(III) Oxide by Methanogens in the Presence and Absence of Extracellular Quinones, *Environ. Microbiol.*, 2002, vol. 4, pp. 115–124.
9. Coleman, M.L., Hedrick, D.B., Lovley, D.R., White, D.C., and Pye, K., Reduction of Fe(III) in Sediments by Sulfate-Reducing Bacteria, *Nature*, 1993, vol. 361, pp. 436–438.
10. Lonergan, D.J., Jenter, H.L., Coates, J.D., Phillips, E.J.P., Schmidt, T.M., and Lovley, D.R., Phylogenetic Analysis of Dissimilatory Fe(III)-Reducing Bacteria, *J. Bacteriol.*, 1996, vol. 178, pp. 2402–2408.
11. Holmes, D.E., Nevin, K.P., and Lovley, D.R., Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB*, and *fusA* genes within the family *Geobacteraceae* fam. nov., *Int. J. Syst. Evol. Microbiol.*, 2004, vol. 54, pp. 1591–1599.
12. Coates, J.D., Phillips, E.J.P., Lonergan, D.J., Jenter, H., and Lovley, D.R., Isolation of *Geobacter* Species from Diverse Sedimentary Environments, *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 1531–1536.
13. Holmes, D.E., Nicoll, J.S., Bond, D.R., and Lovley, D.R., Potential Role of a Novel Psychrotolerant Member of the Family *Geobacteraceae, Geopsychrobacter electrodiphilus* gen. nov., sp. nov., in Electricity Production by a Marine Sediment Fuel Cell, *Appl. Environ. Microbiol.*, 2004, vol. 70, pp. 6023–6030.
14. Vandieken, V., Mumann, M., and Niemann, H., Jorgensen. *Desulfuromonas svalbardens* sp. nov. and *Desulfuromusa ferrireducens* sp. nov., Psychrophilic Fe(III)-Reducing Bacteria Isolated from Arctic Sediments, Svalbard, *Int. J. Syst. Evol. Microbiol.*, 2006, vol. 56, pp. 1133–1139.

15. Robbins, E.I., Bacteria and Archaea in Acidic Environments and a Key to Morphological Identification, *Hydrobiologia*, 2000, vol. 4331, pp. 61–89.
16. Kusel, K., Microbial Cycling of Iron and Sulfur in Acidic Coal Mining Lake Sediments, *Water, Air Soil Pollut.*, 2003, vol. 3, pp. 67–90.
17. Johnson, D.B., Chemical and Microbiological Characteristics of Mineral Spoils and Drainage Waters at Abandoned Coal and Metal Mines, *Water, Air Soil Pollut.*, 2003, vol. 3, pp. 47–66.
18. Ye, Q. and Roh, Y., Carroll, S.L., Blair, B., Zhou, J., Zhang, C.L., Fields, M.W., Alkaline Anaerobic Respiration: Isolation and Characterization of a Novel Alkaliphilic and Metal-Reducing Bacterium, *Arch. Microbiol.*, 2004, vol. 70, pp. 5595–5602.
19. Blum, J.S., Bindi, A.B., Buzzelli, J., Stolz, J.F., and Oremland, R.S., *Bacillus arsenicoselenatis*, sp. nov., and *Bacillus selenitireducens*, sp. nov.: Two Haloalkaliphiles from Mono Lake, California That Respire Oxyanions of Selenium and Arsenic, *Arch. Microbiol.*, 1998, vol. 171, pp. 19–30.
20. Gorlenko, V., Tsapin, A., Namsaraev, Z., Teal, T., Tourova, T., Engler, D., Mielke, R., and Nealson, K., *Anaerobranca californiensis* sp. nov., an Anaerobic, Alkalithermophilic, Fermentative Bacterium Isolated from a Hot Spring on Mono Lake, *Int. J. Syst. Evol. Microbiol.*, 2004, vol. 54, pp. 739–743.
21. Zavarzina, D.G., Zhilina, T.N., Tourova, T.P., Kuznetsov, B.B., Kostrikina, N.A., and Bonch-Osmolovskaya, E.A., *Thermanaerovibrio velox* sp. nov., a New Anaerobic, Thermophilic, Organotrophic Bacterium That Reduces Elemental Sulfur, and Emended Description of Genus *Thermanaerovibrio, Int. J. Syst. Evol. Microbiol.*, 2000, vol. 50, pp. 1287–1295.
22. Burris, R.H., Nitrogen Fixation—Assay Methods and Techniques, *Methods Enzymol.*, 1972, vol. 24B, pp. 415–431.
23. Boulygina, E.S., Kuznetsov, B.B., Marusina, A.I., Tourova, T.P., Kravchenko, I.K., Bykova, S.A., Kolganova, T.V., and Gal'chenko, V.F., A Study of Nucleotide Sequences of *nifH* Genes of Some Methanotrophic Bacteria, *Mikrobiologiya*, 2002, vol. 71, no. 4, pp. 500–508, [*Microbiology* (Engl. Transl.), vol.71, no. 4, pp. 425–432].
24. Lane, D.J, 16S/23S Sequencing, *Nucleic Acid Techniques in Bacterial systematics*, Stackebrandt, E.. and Goodfellow, M., Eds., Chichester, UK: Wiley, 1991, pp. 115–177.
25. Marusina, A.I., Boulygina, E.S., Kuznetsov, B.B., Tourova, T.P., Kravchenko, I.K., and Gal'chenko, V.F., A System of Oligonucleotide Primers for the Amplification of *nifH* Genes of Different Taxonomic Groups of Prokaryotes, *Mikrobiologiya*, 2001, vol. 70, no. 1, pp. 86–91 [*Microbiology* (Engl. Transl.), vol. 70, no. 1, pp. 73–78].
26. Zavarzin, G.A., *Lektsii po prirodovedcheskoi mikrobiologii* (Lectures on Environmental Microbiology), Kolotilova, N.N., Ed., Moscow: Nauka, 2004.
27. Zavarzin, G.A and Zhilina, T.N, Anaerobic Chemotrophic Alkaliphiles, *Journey to Diverse Microbial Worlds*, Seckbach, J., Ed., Kluwer Academic, 2000, pp. 191–208.
28. Zhilina, T.N., Zavarzina, D.G., Kolganova, T.V., Tourova, T.P., and Zavarzin, G.A., "*Candidatus* Contubernalis alkalaceticum," an Obligately Syntrophic Alkaliphilic Bacterium Capable of Anaerobic Acetate Oxidation in a Coculture with *Desulfonatronum cooperativum, Mikrobiologiya*, 2005, vol. 74, no. 6, pp. 800–809, [*Microbiology* (Engl. Transl.), vol. 74, no. 6, pp. 695–703].
29. Kevbrin, V.V., Zhilina, T.N., Rainey, F.A., and Zavarzin, G.A., *Tindallia magadii* gen. nov., sp. nov.: An Alkaliphilic Anaerobic Ammonifier from Soda Lake Deposits, *Curr. Microbiol.*, 1998, vol. 37, pp. 94–100.
30. Garnova, E.S., Zhilina, T.N., Tourova, T.P., and Lysenko, A.M., *Anoxynatronum sibiricum* gen. nov., sp. nov., Alkaliphilic Saccharolytic Anaerobe from Cellulolytic Community of Nizhnee Beloe (Transbailkal Region), *Extremophiles*, 2003, vol. 7, pp. 213–220.