ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 4, pp. 455–467. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

Natronincola ferrireducens sp. nov., and *Natronincola peptidovorans* sp. nov., New Anaerobic Alkaliphilic Peptolytic Iron-reducing Bacteria Isolated from Soda Lakes

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Received July 01, 2008

Abstract—Two novel strains of obligately alkaliphilic (pH 7.5–10.2, optimum pH 8.4–8.8) anaerobic spore-forming rod-shaped bacteria, Z-0511 and Z-7031, were isolated from enrichment cultures obtained from the iron-reducing (Lake Khadyn, Tyva) and cellulolytic (Lake Verkhnee Beloe, Buryatia) bacterial communities, respectively. The organisms ferment peptides and do not ferment proteins and amino acids, with the exception of histidine and glutamate utilized by strain Z-0511. The major fermentation products were acetate and propionate for strain Z-0511 and formate and acetate for strain Z-7031, respectively. Carbohydrates and fermentable organic acids could not serve as substrates, except for pyruvate in the case of strain Z-7031. Nitrogen and sulfur compounds were not utilized as electron acceptors by the strains grown on medium with yeast extract. Strain Z-0511 utilized fumarate, crotonate, and EDTA-Fe(III) as electron acceptors. Anthraquinone-2,6-disulfonate (quinone) and Mn(IV) were utilized by both strains, as well as amorphous ferric hydroxide (AFH), which was reduced to iron sesquioxides and magnetite. The presence of AFH stimulated growth; it enhanced the yield of the fermentation products and changed the quantitative ratios of these products. According to a phylogenetic analysis of the 16S rRNA gene sequences and the phenotypic characteristics of the new strains, they were classified as new species of the genus *Natronincola, Natronincola ferrireducens* sp. nov. Z-0511^T (= VKM B-2402, = DSM 18346) and *Natronincola peptidovorans* sp. nov. Z-7031^T (= VKM B-2503, = DSM 18979).

Key words: soda lakes, alkaliphiles, anaerobes, fermentation, peptides, iron reduction, *Natronincola.*

DOI: 10.1134/S0026261709040092

Non-dissimilatory iron reduction under alkaline conditions was first reported for the alkaliphilic *Tindallia magadiensis* [1] and *Anoxynatronum sibiricum* grown on peptone [2]. The ability to reduce ferric iron of the HTA-Fe(III) complex was demonstrated for cell suspensions of *Bacillus arsenicoselenatis* [3] and for the halotolerant alkaliphilic *Bacillus* sp. in the course of fructose fermentation [4]. The thermoalkaliphilic bacterium *Anaerobranca californiensis* reduced iron in the presence of peptides [5]. Dissimilatory iron reduction with soluble iron complexes was first reported for *Alkaliphilus metalliredigens* isolated from an alkaline pond in the exploitation zone of a boron deposit (United States) [6].

The ability of microorganisms to reduce amorphous and weakly crystalline iron oxides under alkaline conditions was considered doubtful [6] due to the low activity of Fe(III) under alkaline conditions. However, recent studies of iron reduction in the bottom sediments of African and Asian soda lakes demonstrated that reduction of amorphous ferric hydroxide (AFH) is possible under these conditions. The first obligately alkaliphilic dissimilatory iron reducer, *Geoalkalibacter ferrihydriticus*, which reduces AFH utilizing acetate and some other organic acids as electron donors, was isolated, described, and validated [7].

According to phylogenetic analyses, the majority of known species of alkaliphilic anaerobes capable of iron reduction are low G + C gram-positive bacteria of the family *Clostridiaceae* [1, 2, 5], which utilize iron oxides in the course of so-called facilitated fermentation.

This paper presents the taxonomic description of two novel strains of the alkaliphilic representatives of cluster XI of the family *Clostridiaceae*, which are capable of AFH reduction in the course of peptide fermentation with the resulting formation of iron sesquioxides and magnetite.

MATERIALS AND METHODS

Source of isolation. Strain Z-7031 was isolated from the cellulose-degrading anaerobic microbial com-

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munity in which the previously isolated *Clostridium alkalicellulosi* acted as a cellulolytic microorganism [8]. The source for the community development was a sample from the bottom sediment of a coastal lagoon of the soda lake Verkhnee Beloe (Buryatia) (mineralization, 7.5 g/l; pH 10). Strain Z-0511 was a component of the anaerobic iron-reducing microbial community from which *Geoalkalibacter ferrihydriticus* had previously been isolated [7]. This community was risen from a sample of anoxic bottom sediment from Lake Khadyn (Tyva); the lake water has a total mineralization of 17.0 g/l and a pH of 9.5.

Cultivation conditions. For selective enrichment and isolation of the peptolytic strains, we used the mineral media prepared, under strictly anaerobic conditions in the nitrogen atmosphere, in accordance with the mineral composition of the lakes as described in [7, 8]. The optimized medium (pH 9.0) for both strains contained the following (g/l): KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 0.5; KCl, 0.2; NaCl, 1.0; Na_2CO_3, 3.0; $NaHCO_3$, 10.0; yeast extract (Difco), 3.0; and trace element solution [9], 1 ml/l. N_2 was used as the gas phase. Inoculated media were incubated in a thermostat at 35°C for a week.

To determine the substrates used for catabolism, they were added in a concentration of 3 g/l; amino acids and alcohols were added in the concentrations of 2 g/l and 5 ml/l, respectively. Carbohydrates were added to the alkaline medium as concentrated sterile water solutions immediately before inoculation.

Physiological characteristics. The electron acceptors were added to sterile NH_4Cl-free medium as concentrated solutions to the following concentrations (mM): $Na_2S_2O_4$, 1.0; Na_2SO_3, 2 and 20; $Na_2S_2O_3 \cdot 5H_2O$, 10; Na_2SO_4, 20; $NaNO_2$, 2 and 10; $NaNO_3$, 10; quinone, 20; Mn(IV) (in the form of artificially synthesized MnO_2), 25; EDTA-Fe(III), 20 + cysteine (0.3 g/l); AFH, 90 in terms of Fe(III) + cysteine, 0.3 g/l; Fe(III) citrate, 5.0; S°, 2% (wt/vol); crotonate, 10; and fumarate, 5.0. The AFH suspension and EDTA-Fe(III) solution were prepared as described in [7].

The effects of pH, NaCl concentrations, and temperature on cell growth, as well as the dependence of growth on Na^+ and Cl^- ions, were assessed as described previously [8].

The effect of carbonates was determined by replacing them by equimolar amounts of Na_2SO_4 and maintaining the pH level at 8.1 or 9.7 with Tris buffer or 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (50 mM), respectively. The growth ranges and optimal concentrations of carbonate were determined by varying the carbonate concentrations at the constant $Na_2CO_3/NaHCO_3$ ratio of 0.3. By multiplying the $NaHCO_3$ concentration values (0.1; 0.5; 1.0; 5.0; 10; 20; 30; 40; 50; and 100 g/l) by 0.3, we obtain the Na_2CO_3 concentration values. After sterilization, the pH of all media varied insignificantly.

The capacity for microaerophilic growth and O_2 utilization was assessed by the addition of O_2 (0.2; 0.4; 1.4; 2.3; 4.2; 7; and 9.3%) to the medium prepared under strictly anaerobic conditions in the atmosphere of nitrogen without a reducing agent. Bacterial biomass (measured as OD_{600} on a spectrophotometer) and O_2 concentration (measured on a gas chromatograph) were determined at the beginning and end of growth. Catalase activity was assayed by monitoring the formation of gas bubbles upon addition of a 3% hydrogen peroxide solution to the cell suspension.

Analytical methods. Growth rate was determined from the optical density of the cultures measured directly in Hungate tubes at 600 nm on a Specol-10 spectrophotometer (Jena, Germany) or a UNICO-2100 spectrophotometer. The concentrations of hydrogen, oxygen, and nitrogen were determined on an LHM-80 gas chromatograph (Russia) equipped with a katharometer detector. The contents of organic fermentation products were determined by HPLC on a Staier high pressure chromatograph (Russia) equipped with a refractometer detector. The separation was carried out on an Aminex HPX-87H column (BioRad, United States); 5 mM H_2SO_4 was used as an eluent. The content of dissolved sulfide was determined colorimetrically by the formation of methylene blue. The fatty acid (FA) composition of microbial lipids was determined on a Microbial Identification System (Sherlock) chromatograph (MIDI Inc., Newark, United States) according to the procedure described in [10]. The separated fatty acids were identified using an Agilent Technologies AT-5971 SMART mass spectrometer.

Utilization of sulfur-containing electron acceptors was assessed from the production of hydrogen sulfide (determined colorimetrically by the formation of methylene blue). Reduction of nitrogen compounds was determined from N_2 production in the media with argon as the gas phase. The rate of NH_4 production from NO_2^- and NO_3^- was determined using the Nessler's reagent. The rate of iron oxide reduction was determined by the colorimetric reaction with ferrocene as described in [11].

The contents of iron-containing minerals were determined by Mössbauer spectroscopy [12].

Morphology. The cell morphology in live specimens was examined under a Zetopan phase-contrast microscope (Reichert, Austria). Ultrathin sections and whole cells were contrasted with 1% phosphotungstic acid to reveal flagella and examined under a JEM-100C electron microscope (Japan).

DNA analysis. The DNA G+C content was determined from the melting temperatures of DNA as described in [2].

Isolation of DNA and amplification and sequencing of the 16S rRNA genes. Isolation and purification of the DNA preparations were carried out according to the previously described procedure [2].

Amplification and sequencing of the 16S rRNA genes were carried out using primers universal for bacteria [13]. Sequencing of the PCR products was performed by the Sanger's method on an automatic ABI 3730 sequencer (Applied Biosystems, United States) using a Big Dye Terminator sequencing kit (version 3.1) according to the manufacturer's instructions.

Phylogenetic analysis of the 16S rRNA gene sequences. The sequences were edited using the BioEdit sequence alignment editor [http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html]. The primary comparison of the de novo obtained sequences with sequences from the GenBank database was performed using the NCBI BLAST software package [http://www.ncbi.nlm.nih.gov/blast]. The newly determined nucleotide sequences of the 16S rRNA genes were aligned with the corresponding sequences of the most closely related bacteria using the CLUSTALW v. 1.75 software package. The phylogenetic tree was constructed using the methods implemented in the TREECONW [http://bioc-www.uia.ac.be/u/yvdp/treeconw. html] software package.

Deposition of the nucleotide sequences. The obtained nucleotide sequences of the 16S rRNA genes of strains Z-7031 and Z-0511 were deposited in the GenBank under accession numbers EF382661 and EU878275, respectively.

RESULTS

Isolation. Strain Z-7031 from Lake Verkhnee Beloe (Buryatia) was isolated from the cellulolytic microbial community developed on microcrystalline cellulose supplemented with 0.2 g/l of yeast extract and containing a minimum amount of bacterial forms after heating at 90°C for 20 min [8]. The culture contained only morphologically different spore-forming rod-shaped cells. The presence of strain Z-7031was revealed by inoculation of a peptone-containing medium, where tenfold dilutions produced its growth up to dilution 10^{-6}. However, on the peptone-containing medium, rods of different lengths and diameters were detected. Some cells produced terminal spores; chains of five to six cells and thin long filaments occurred. Thus, the population was heterogenous. The pure culture of the strain Z-7031 was obtained by inoculation of agarized (2% agar) medium with peptone and yeast extract, isolation of single colonies in roll-tubes, and then by the serial dilutions method in liquid medium.

Strain Z-0511 was isolated from the iron-reducing community obtained from a Lake Khadyn (Tyva) sample on medium that contained acetate (3 g/l) as an electron donor, AFH as an electron acceptor, and yeast extract (100 mg/l) as a source of growth factors [7]. Inoculation of the enrichment culture into medium with yeast extract (3 g/l), AFH, and quinone (20 mM) resulted in intense quinone reduction with the development of thin spore-forming rods of different sizes. Isolation of the pure culture of strain Z-0511 was carried out on an agarized medium with yeast extract by a procedure similar to that used for strain Z-7031 (see above).

The purity of the cultures was confirmed by microscopic examinations and partial sequencing of the their 16S rRNA genes using various PCR primers.

Morphology. Under a light microscope, young cultures of strains Z-7031 and Z-0511 have similar cell morphology. The cells are thin straight or slightly curved rods, 1.5–4.0 μm long and 0.2–0.5 μm in diameter (Figs. 1 and 2). Sometimes they occur at an angle to each other. The characteristic traits of these cells are their arrangement in the form of palisades (Figs. 1a and 2a) and the presence of capsules outside the cell wall (Figs. 1b and 2b).

At advanced developmental stages of strain 7031, long (up 10–20 μm) cells with terminal spherical spores (0.5 μm in diameter) were detected (Fig. 2a). Minicells (0.15 μm in diameter) were sometimes observed at the cell poles . Only in young cultures of strain Z-7031 were the cells motile by means of one or two lateral flagella (Fig. 1b). In the case of strain Z-0511, the cells were motile, although flagella were not detected (Fig. 2b). The structure of the cell wall of both strains is of the gram-positive type (Figs. 1c and 2c).

Under a light microscope, strain Z-0511 cells are of medium optical density and exhibit poor adsorption to glass. The strain reproduces by cell division (often nonuniform) with formation of a septum followed by the splitting and sliding of the separated daughter cells (Fig. 2c).

Strain Z-7031 forms white disk-shaped colonies (0.1–1 mm in diameter) with an asymmetrically stretched edge and fine-grained moire surface. Strain Z-0511 forms light beige disk-shaped colonies (0.2–0.4 mm in diameter) with even edges; the colonies are located within the agar layer.

Growth characteristics. The strains were obligate alkaliphiles: they grew within the pH range of 7.5–10.2 with an optimum at pH 8.4–8.8 for strain Z-7031 and a sharply pronounced optimum at pH 8.4 for strain Z-0511. At pH lower than 6.8 or higher than 10.2, no growth occurred (Fig. 3).

The organisms did not require NaCl. However, the strains were halotolerant: strain Z-0511 grew at a salinity of up to 60 g/l NaCl with an optimum at 5–7 g/l; strain Z-7031 grew at a salinity of up to 70 g/l NaCl with an optimum at 10–25 g/l. The strains did not exhibit obligate requirement for carbonate ions. Replacement of carbonates with sodium sulfate at the optimal Na^+ concentration growth was possible if the optimal pH range was maintained with Tris or CAPS buffers. However, the optimum $Na_2CO_3/NaHCO_3$ concentrations for the strains Z-7031 and Z-0511 were 6.0/20.0 and 3.0/10.0 g/l, respectively (Fig. 4).

With respect to temperature, the organisms were typical mesophiles growing within the temperature

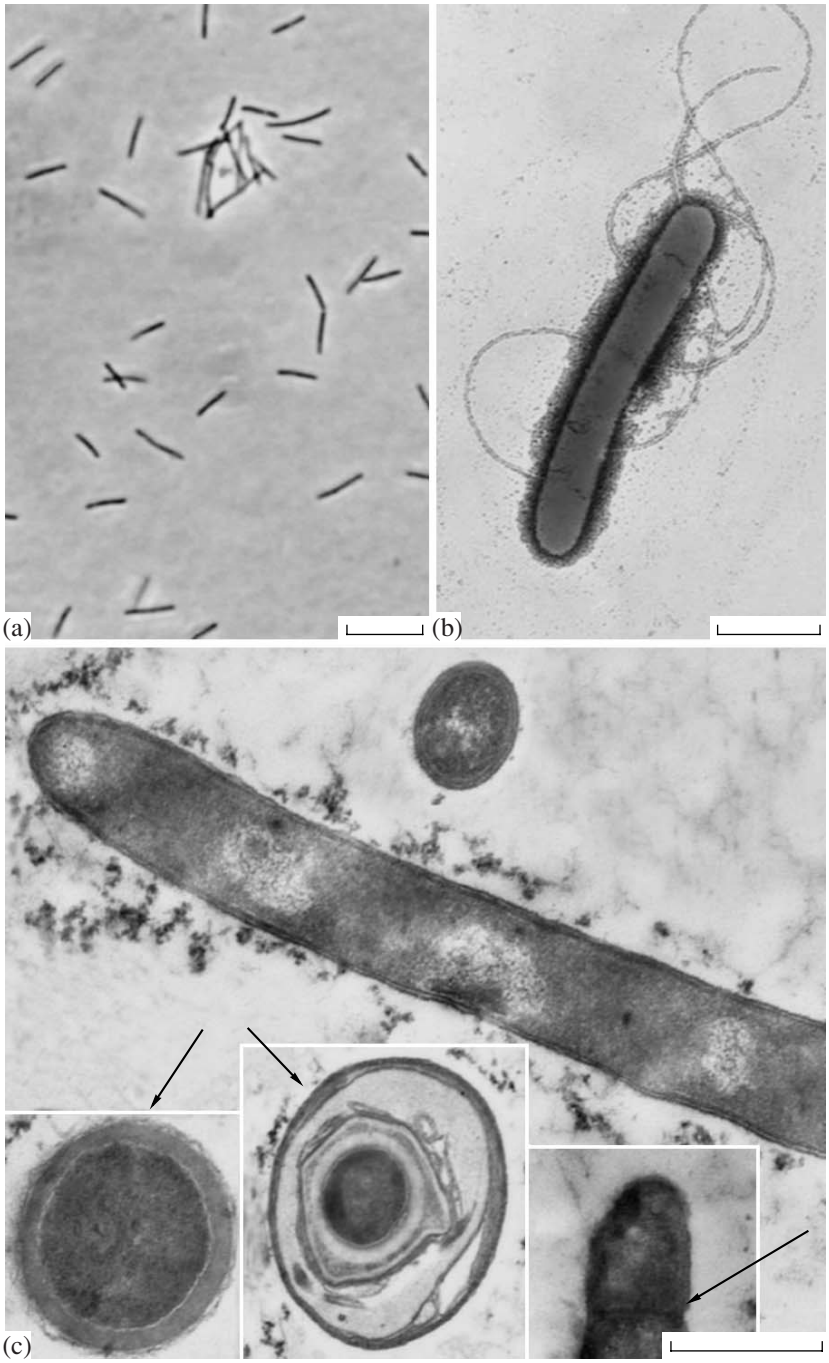

Fig. 1. Cell morphology of strain Z-7031. Light microscopy (a): vegetative cells and cells with spores. Scale bar, 10 μm. Electron microscopy (b, c): cell with lateral flagella; scale bar, 0.5μm (b); longitudinal ultrathin section of a cell with the cell wall structure typical of gram-positive bacteria (c). Arrows indicate spores at different stages of development and cell septation.

range of 15–40°C with an optimum of 35–37°C. At 15°C, growth was weak; the duration of the lag phase extended to 4 days.

The new isolates were anaerobes; oxygen was not used as an electron acceptor. They did not grow under aerobic or microaerobic conditions in the presence of more than 0.2% O_2; thus, they were aerotolerant microorganisms. In the presence of oxygen, the organisms did not utilize it and carried out anaerobic fermentation. During anaerobic growth, the strains were able to grow in the absence of reducing agents; however, the addition of sulfide, thioglycolate, or cysteine in concentrations of 0.3–0.5g/l significantly decreased the duration of the lag phase.

Strains Z-7031 and Z-0511, isolated on media with peptone and yeast extract, turned out to be strictly lim-

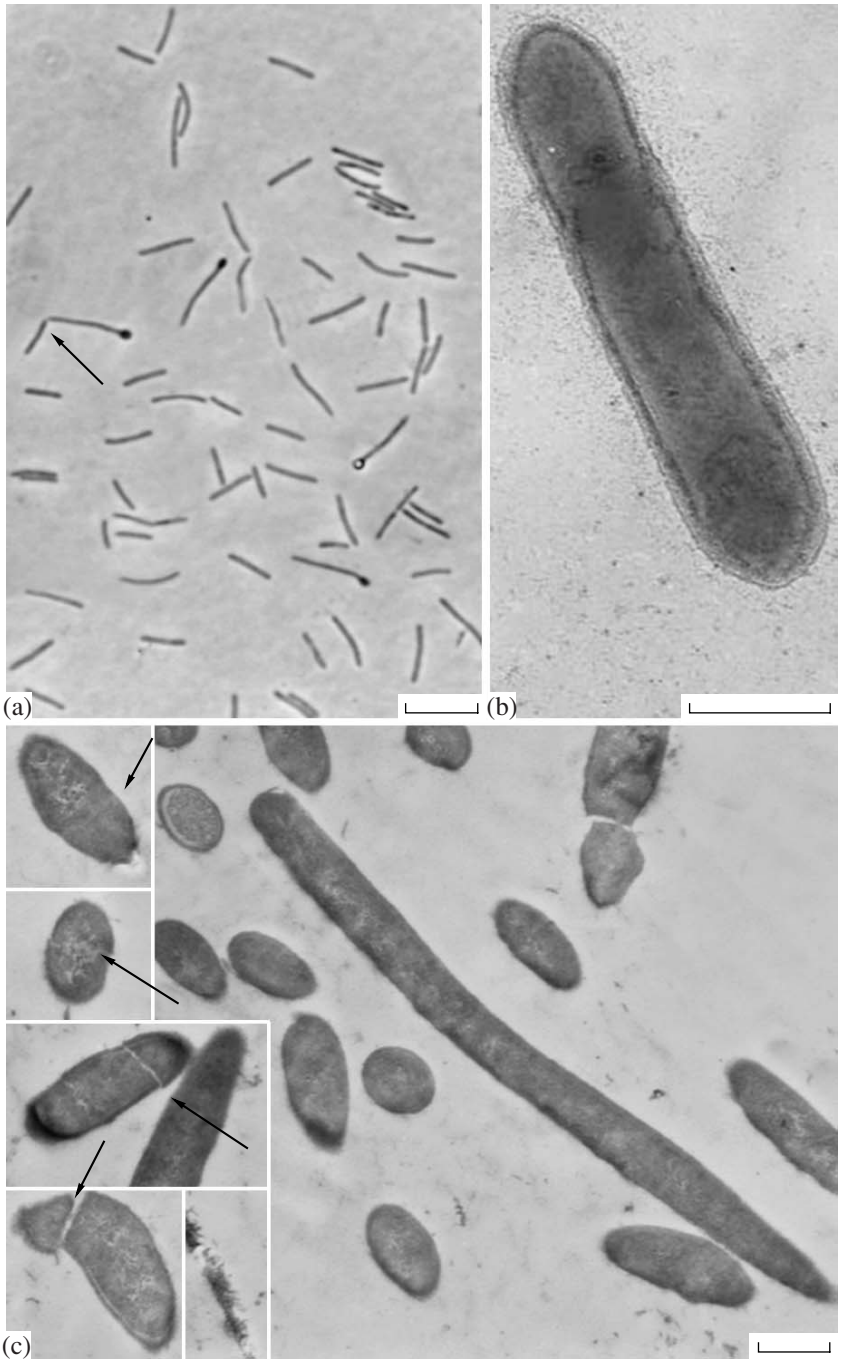

Fig. 2. Cell morphology of strain Z-0511. Light microscopy (a): vegetative cells and cells with spores; scale bar, 10 μm. Electron microscopy (b, c): cell in its dense capsule; scale bar, 0.5 μm (b); longitudinal ultrathin section of a cell with the cell wall structure typical of gram-positive bacteria (c). Arrows indicate spores at different stages of development and cell septation.

ited in nutritive capacities by a number of extractive protein substrates (Bacto yeast extract, BBL trypticase, Bacto Soytone, Bacto tryptone, meat extract, Bacto peptone, and Difco Casamino acids). In addition, strain Z-7031 utilized pyruvate, whereas strain Z-0511 utilized histidine and, to a small extent, glutamate. The best growth was noted on yeast extract, whereas the rate of peptone utilization was low. The growth rate of the new strains was proportional to the amount of the nitrogen substrate added to the medium; the organisms were not oligotrophic. During growth on individual amino acids, vitamins or yeast extract (50 mg/l) were required.

In the presence of vitamins or additional 50 mg/l of yeast extract, no growth occurred on asparagine, glutamate (Z-0511 was capable of weak growth), glutamine, proline, ornithine, threonine, arginine,

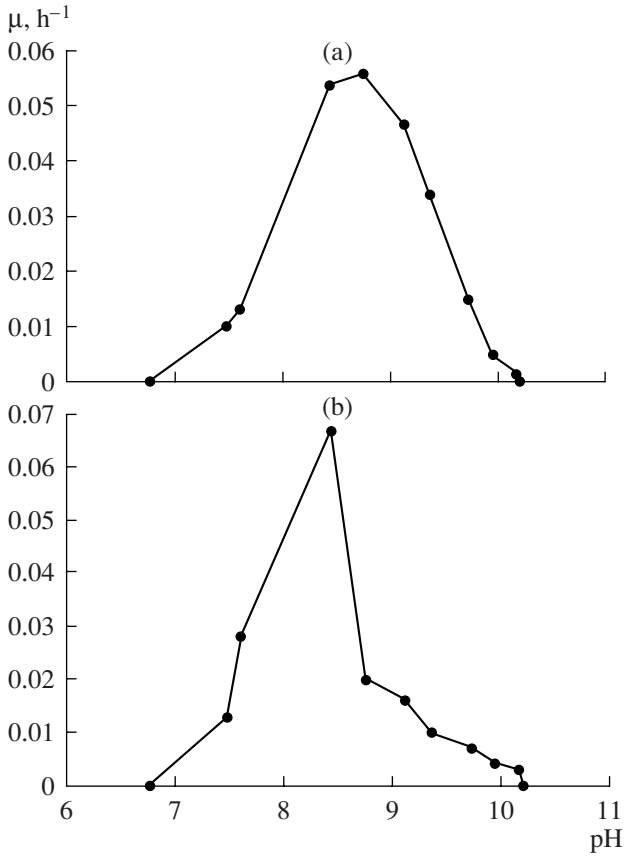

Fig. 3. Growth rate of strains Z-7031(a) and Z-0511(b) as dependent on pH value.

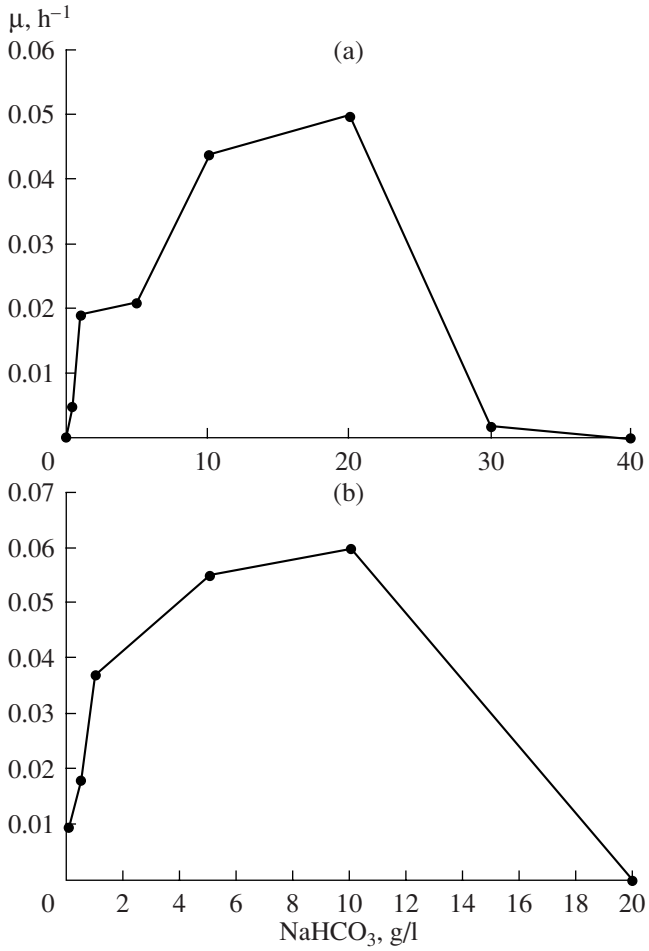

Fig. 4. Growth rate of strains Z-7031(a) and Z-0511(b) as dependent on carbonate concentrations. The concentration of Na_2CO_3 at each point is equal to the $NaHCO_3$ concentration multiplied by 0.3.

lysine, valine, histidine (Z-0511 showed good growth), β-alanine, glycine, serine, leucine, methionine, tyrosine, tryptophan; proline + valine, proline + alanine, proline + isoleucine, proline + leucine, proline + histidine, glycine + valine, glycine + alanine, glycine + isoleucine, glycine + leucine, glycine + histidine; pectin, xylan, casein, albumin, gelatin; sugars (cellobiose, arabinose, galactose, glucose, xylose, lactose, maltose, mannose, sucrose, sorbose, rhamnose, ribose, trehalose, fructose, fucose, raffinose, melibiose, N-acetyl-D-glucosamine, inositol, and sorbitol); alcohols (ethanol and methanol); organic acids: lactate, pyruvate (Z-7031 showed growth); glycerol, propionate, malonate, betaine, glycolate, oxalate, citrate, fumarate, crotonate, uracil, ethylene glycol, or 2,3-butanediol.

Both strains are incapable of growth by proteolysis of the cell biomass of *Euhalotheca, Microcoleus, or Spirulina* or Gaprin (dried *Methylococcus* biomass).

Acetate and formate (1 : 1) were the main products formed in the course of yeast extract fermentation by strain Z-7031, while strain Z-0511 produced acetate and propionate (Figs. 5a and 6a). Formate was a minor component produced by strain Z-0511, and strain Z-7031 produced minor amounts of propionate, isobutyrate, and H_2. During growth on pyruvate, strain Z-7031 produced acetate and formate (1 : 1), as well as H_2.

Growth in the presence of external electron acceptors. When studying the growth of the isolates in the presence of yeast extract as an electron donor and various electron acceptors, it was demonstrated that the organisms did not reduce S^0 and oxides of sulfur (SO_4^{2-}, SO_3^{2-}, and $S_2O_3^{2-}$) or nitrogen. Growth in the presence of NO_3^- (10 mM) and NO_2^- (2 mM) was comparable to that in the control; however, 10 mM NO_2^- completely inhibited growth of both strains. Only strain Z-0511 utilized fumarate and crotonate as electron acceptors; 4.6 mM succinate and 6.4 mM acetate from yeast extract were formed from 5 mM fumarate; 11.6 mM acetate and 1.76 mM butyrate were formed from 10 mM crotonate, with a twofold increase in the acetate production as compared to yeast extract fermentation and absence of formate production.

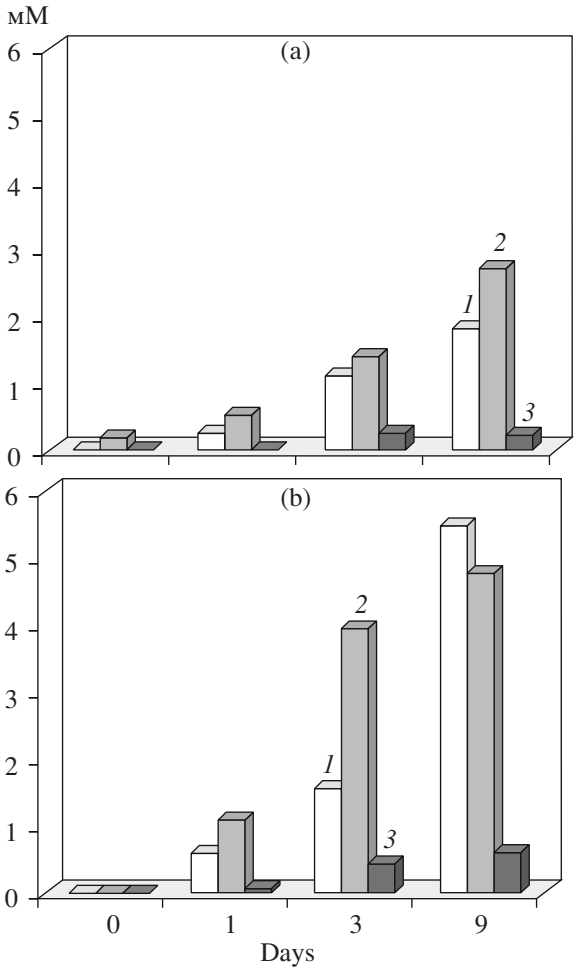

Fig. 5. Effect of AFH on the product formation by strain Z-7031: AFH-free medium (a) and AFH-containing medium (b). Concentrations (mM): formate (*1*); acetate (*2*); and propionate (*3*).

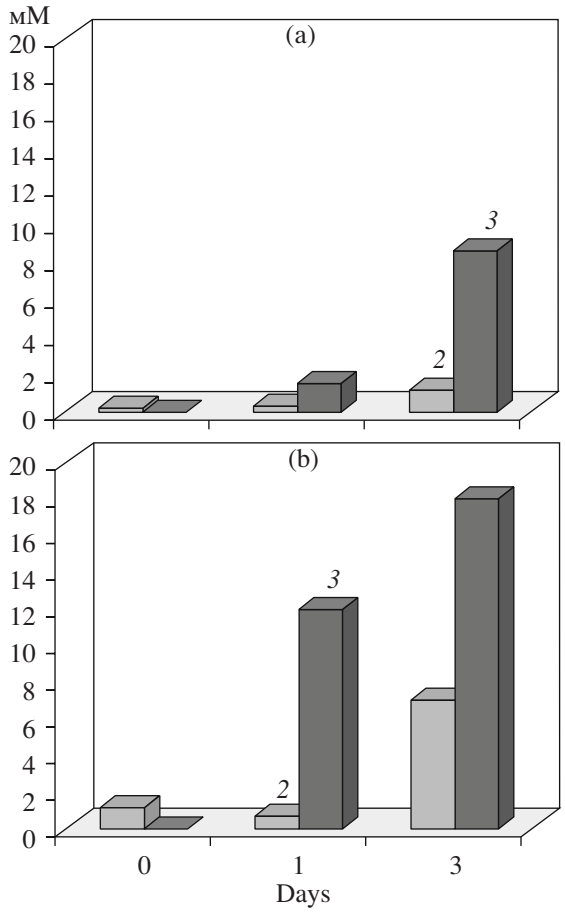

Fig.6. Effect of AFH on the product formation by strain Z-0511: AFH-free medium (a) and AFH-containing medium (b). Concentrations (mM): acetate (*2*) and propionate (*3*).

The strains reduced AFH with the resulting formation of a black solid sediment consisting of iron sesquioxides and magnetite (strain Z-7031). Among the soluble forms of iron, strain Z-0511 reduced the EDTA-Fe(III) complex and did not reduce Fe(III) citrate. Both strains reduced quinone and Mn(IV). The reduction of quinone was accompanied by a change in the medium pigmentation (from light yellow to deep brown), easily notable against the background of abiogenic control. The reduction of MnO_2 was accompanied by changes in the sediment pigmentation (from deep brown to white) due to Mn(II) formation.

The addition of 90 mM AFH in the presence of 0.3 g/l of cysteine resulted in a doubling or tripling of the yield of the fermentation products on yeast extract (Figs. 5 and 6), as well as in the production (by day 9 of incubation) of 18.1 and 12.6 mM Fe(II) by strains Z-7031 and Z-0511, respectively.

Sensitivity to antibiotics. The growth of strains Z-7031 and Z-0511 was not affected by the addition of 100 mg/l of ampicillin, kanamycin, chloramphenicol, penicillin, or neomycin, whereas the addition of streptomycin, rifampicin, vancomycin, novobiocin, or bacitracin completely inhibited growth.

Analysis of the fatty acid composition. Table 1 shows the results of the comparative fatty acid analysis of the strains under study. It was found that $C_{16:1\omega7c}$, $C_{16:0}$, $C_{18:1\omega9}$, $C_{14:0}$, and $C_{18:0}$ constituted 70.5 and 72.5% of the total fatty acids of the lipid membranes of strains Z-7031 and Z-0511, respectively.

Phylogenetic analysis. According to the phylogenetic analysis of the 16S rRNA gene sequences, strains Z-7031 and Z-0511 clustered with strains of cluster XI of low G + C gram-positive bacteria of the family *Clostridiaceae*. Comparative analysis of the sequences of the 16S rRNA genes of strains Z-7031 and Z-0511 revealed a structural difference between them consisting in the presence of an unusually long insertion (approximately 120 nucleotides) in the variable region of strain Z-0511 16S rRNA (at the *E. coli* position 80). With this insertion, the difference between the nucleotide sequences of these strains was 6.0%; without the

Table 1. Fatty acid composition of strains Z-7031^T and Z-0511^T (% of the total fatty acids)

Fatty acid	Z-7031	Z-0511	Fatty acid	Z-7031	Z-0511
$C_{9:0}$	–	0.15	**$C_{16:0}$**	**15.37**	**14.55**
$C_{10:0}$	0.27	0.33	C_{i16a}	0.87	1.25
$C_{11:0}$	–	0.33	$C_{16:1a\ cis9}$	1.03	2.66
$C_{12:0}$	1.20	2.26	$C_{16:1a\ trans9}$	–	0.38
C_{i13}	–	0.68	C_{16a}	0.17	3.65
$C_{13:0}$	2.80	1.95	C_{i17}	–	0.31
C_{i14}	0.43	–	C_{a17}	–	1.27
$C_{14:1\omega5}$	0.41	1.08	$C_{17:1\omega6}$	1.29	0.74
$C_{14:1\omega3}$	0.60	0.49	$C_{17:0}$	0.77	0.53
$C_{14:0}$	**11.97**	**6.44**	**$C_{18:1\omega9}$**	**13.58**	**16.40**
C_{i15}	0.27	0.50	$C_{18:1\omega7}$	2.08	2.13
C_{a15}	0.58	0.90	**$C_{18:0}$**	**4.42**	**5.31**
$C_{15:1\omega8}$	4.10	0.74	$C_{18:1\omega9a}$	–	2.64
$C_{15:1\omega6}$	3.40	0.22	$C_{18:1\omega7a}$	–	0.34
$C_{15:0}$	**9.28**	**1.17**	$C_{18:0a}$	–	0.41
C_{i16}	–	0.19	$C_{20:0}$	–	0.25
$C_{16:1\omega7c}$	**25.11**	**29.75**	Total, %	100	100

insertion, it was 5.5%. Both strains occupied separate branches within the *Clostridium felsineum* subgroup [14]; the similarity levels between their 16S rRNA gene sequences and those of the closest species did not exceed 95%, confirming the affiliation of strains Z-7031 and Z-0511 with new species. In addition, the similarity level between their 16S rRNA gene sequences, like between them and those of the closest relatives, was considerably below 97% [15], the value that previously dictated necessity of DNA–DNA hybridization (recently, this value has been reduced to 98.7–99% [16]). At the same time, the similarity levels between the 16S rRNA gene sequences of strains Z-0511 and Z-7031 and those of *C. felsineum*, *C. formicoaceticum* [17, 18] and *Natronincola histidinovorans* [19] were almost the same (93.2–94.8%, 93.7–95.1%, and 92.2–94.1%, respectively). The 16S rRNA gene similarity level between the new strains and *Tindallia magadiensis* [1] was significantly lower (88.5–90%); the levels of similarity with some other members of the genus *Clostridium*, including alkaliphilic ones, were also low (85.6–88.8%). The phylogenetic tree in Fig. 7 shows that strains Z-7031 and Z-0511 belong to the *Clostridium felsineum* phylogenetic subgroup of cluster XI.

The DNA G + C contents of strains Z-7031 and Z-0511 were 35.5 ± 0.3 and 35.3 ± 0.5 mol %, respectively.

DISCUSSION

The peptide-fermenting strains isolated by us from the cellulolytic and iron-reducing communities were initially grown on the medium to which yeast extract (200–100 mg/l) was added as a growth factor and which contained elective substrates for anaerobic alkaliphiles with specific functions (cellulose for *Clostridium alkalicellulosi* [8] or acetate and AFH for *Geoalkalibacter ferrihydriticus* [7]). However, this low concentration of yeast extract proved sufficient for the stable growth of the studied strains as satellites within these functionally different communities.

The new isolates are organoheterotrophs and utilize the nitrogen-containing compounds of the proteolytic pathway. They do not utilize carbohydrates or nitrogen-free fermentation products as substrates; however, strain Z-7031 utilizes pyruvate, a central metabolism substrate. The strains do not possess proteases required for hydrolysis of polymeric compounds (albumin and gelatin) algal biomass or Gaprin. They are capable of weak growth on the amino acid mixture (Casamino acids) and do not carry out the Stickland reaction. They do not ferment individual amino acids, except that strain Z-0511 ferments histidine and, to a lesser extent, glutamate. The limited spectrum of the substrates utilized by the new isolates is possibly due to the peculiarities of their transport systems. Thus, peptides are to be considered their trophic specialization in the microbial community.

Judging from the fermentation products formed by strain Z-7031, it has the same fermentation type as the freshwater saccharolytic *Clostridium formicoaceticum*

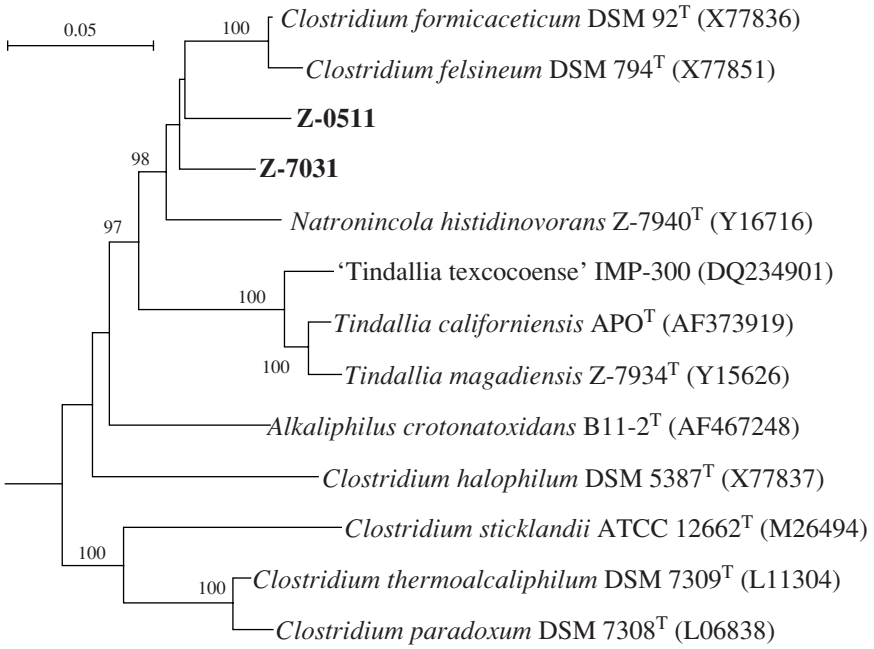

Fig. 7. Phylogenetic position of the new strains Z-7031 and Z-0511 among members of the cluster XI of low G + C gram-positive bacteria. The phylogenetic tree was constructed on the basis of the results of comparative 16S rRNA gene analysis. Bar corresponds to 5 nucleotide substitutions per 100 nucleotides.

(corrig. *C. formicaceticum*) [18]; strain Z-0511 has the same fermentation type as the peptolytic acetogens *Natronoincola* (corrig. *Natronincola*) [19] and *Tindallia* [1].

The ecophysiological characteristics mark the new isolates as inhabitants of the bottom sediments of continental steppe water bodies. The studied microorganisms were isolated from the geographically distant (Republic of Tyva and Southeastern Transbaikal Region) but geochemically similar sources: bottom sediments of the low-mineralization steppe soda lakes. Their pH growth range, temperature dependence, low salinity requirement, and ability to grow on carbonate-containing media mark them as obligately alkaliphilic halotolerant organisms (Table 2). With respect to their osmotic characteristics, the organisms are inhabitants of moderately mineralized waters with a relatively low (less than 70 g/l) upper limit of mineral salt content. The narrow osmotolerance range indicates that the isolates do not belong to inhabitants of lakes that intermittently dry up or of soda salt marshes. The lack of the obligate requirement for chloride and carbonate ions does not permit classification of the studied strains as halophiles or natronophiles.

The strains are moderately aerotolerant microorganisms. The presence of catalase allows them to grow at an O_2 content below 0.2%. However, in the presence of oxygen, the organisms do not utilize it as an electron acceptor and perform anaerobic fermentation, which permits us to assign them to obligate anaerobes. Utilization of some organic and inorganic electron acceptors, such as fumarate, crotonate, quinone, AFH, Mn(IV), and EDTA-Fe(III) by strain Z-0511, as well as of quinone, Mn(IV), and AFH by strain Z-7031, indicates that these organisms are able to switch between the fermentative and the respiratory types of metabolism.

The ability of the new isolates to reduce AFH is worth further discussion. Few alkaliphiles capable of iron reduction are currently known. Despite the fact that iron reducers are widespread and have been found in various habitats [7], iron reduction under alkaline conditions has not been studied adequately due to the very low iron solubility under these conditions. All the experiments with alkaliphilic microorganisms were carried out using soluble iron complexes [1–6]. We have previously investigated dissimilatory iron reduction on acetate and AFH using, as an example, the bottom sediments of four soda lakes that differed in salinity and alkalinity levels. The results indicated that, under these conditions, iron reduction was possible and involved insoluble iron compounds. The alkaliphilic acetate-oxidizing iron reducer *Geoalkalibacter ferrihydriticus* reduced AFH to magnetite and siderite with the resulting formation of Fe(II) (40 mM) from the Fe(III) (90mM) of the AFH added to the medium [7]. In the presence of yeast extract, the new alkaliphilic obligately anaerobic bacteria reduced AFH with the resulting formation of 13–20% Fe(II) from the total Fe(III) of the AFH added to the medium, confirming the previously obtained results [7] and suggesting that, among various groups of alkaliphilic microorganisms, iron reduction may be a common function, as it is among neutrophilic bacteria.

Table 2. Differentiating characteristics of the studied strains and their closest relatives from the family *Clostridiaceae* cluster XI

Characteristics	Z-7031^T	Z-0511^T	*N. histidinovorans* Z-7940^T [18]	*C. felsineum* DSM 794 [16]	*C. formicaceticum* QYMF^T [17]
Morphology: slightly curved rods	+	+	+	+	+
Size, μm	0.2–0.5 × 1.5–10	0.2–0.5 × 1.5–8.0	0.7–1.0 × 2.0–6.0	0.5–1.3 × 3.1–25.7	1.2–2.0 × 5.0–12.0
Flagella	+	–	+	+	+
Growth at pH < 7.0	–	–	–	+	+
Growth at pH 8.5–10.0	+	+	+	–	–
Optimum pH	8.4–8.8	8.4	9.4	7.0	7.5–8.0
NaCl growth range, g/l	0–70	0–60	40–160	0	0
Acceptors	AFH, quinone, Mn(IV)	AFH, EDTA-Fe(III), quinone, fumarate, crotonate, Mn(IV)	ND	ND	ND
Substrates:					
tryptone or peptone	±	±	±	–	–
yeast extract	+	+	+	–	–
casamino acids	±	±	+	ND	ND
sugars	–	–	–	+	+
pectin	–	–	–	+	+
histidine	–	+	+	ND	ND
lactate	–	–	–	–	+
pyruvate	+	–	±	ND	+
citrate	–	–	–	ND	ND
glutamate	–	±	+	ND	ND
Major fatty acids	$C_{16:0\omega7c}$, $C_{16:0}$, $C_{18:1\omega9}$, $C_{14:0}$, $C_{15:0}$, $C_{18:0}$	$C_{16:0\omega7c}$, $C_{16:0}$, $C_{18:1\omega9}$, $C_{18:0}$, $C_{14:0}$	$C_{16:0}$, $C_{16:0\omega9}$, aldehyde C_{16}, $C_{14:0}$	ND	ND
Major fermentation products	Acetate, formate	Acetate, propionate	Acetate, NH_3	Acetate, butyrate	Acetate
Minor fermentation products	H_2, propionate, isobutyrate	Formate	Formate	Butanol	Formate
DNA G+C content, mol %	35.5 (T_m)	35.3 (T_m)	31.9 (T_m)	26.0 (T_m)	34 (T_m)
Isolation source	Low-mineralization soda lake Verkhnee Beloe (Buryatia, Russia)	Low-mineralization soda Lake Khadyn (Tyva, Russia)	Soda Lake Magadi (Kenya)	Gutter mud, sewage waters (Göttingen, Germany)	Sewage waters, stagnant river water (United States)

In the presence of AFH, both new isolates form two to three times more products (Figs. 5 and 6). The difference between the products formed during growth in the presence and absence of AFH attracts attention. In the cultures of strain Z-7031 grown with AFH, propionate was detected on the first day of the experiment; on the ninth day, formate, rather than acetate, was found to be the major product (Fig. 5). In the case of strain Z-0511 grown on the AFH-containing medium, a large propionate release was detected (12 mM in comparison to 1.53 mM on a medium with yeast extract only). On the third day of the experiment, the cells produced 7mM acetate and 18 mM propionate on the AFH-containing medium, as compared to 1.2 and 8.7 mM acetate and propionate, respectively, produced on the iron-free medium (Fig. 6). Thus, the studied alkaliphilic bacteria are capable of intense iron reduction.

The new isolates, despite their similar ecophysiological characteristics, differ in the spectrum of substrates and electron acceptors, products of metabolism, and in the FA lipid composition (Table 2). The fatty acid profiles of the studied strains were similar (Table 1); however, the content of $C_{15:0}$ (pentadecanoic acid) in the cells of strain Z-7031 was considerably higher, 9.28% as compared to 1.17% for strain Z-0511. In both strains, *cis*-9-hexadecenoic acid ($C_{16:1\omega7c}$) prevailed (25.11–29.75%). This acid is predominant in the fatty acid profile of the dissimilatory alkaliphilic iron

reducer *"Alkaliphilus metalliredigens"* [6], and the proportion of this fatty acid in the phylogenetically distant dissimilatory iron reducers *Shewanella algae* and *Geobacter metallireducens* was similar to that observed in *"A. metalliredigens"* grown with Fe(III). It was suggested that $C_{16:1\omega 7c}$ is a common constituent of membranes of metal-reducing bacteria [6]. The fact that this amino acid is predominant in the FA profiles of *T. magadiensis* [19], *Alkaliphilus peptidofermentans* [21], and strains Z-7031 and Z-0511, capable of reducing Fe(III), is in accordance with this assumption. These findings indicate the possibility of using this acid as a marker for metal reduction.

Phylogenetically, the new isolates belong to the *Clostridium felsineum* phylogenetic subgroup of cluster XI of low G + C gram-positive bacteria of the family *Clostridiaceae*, where they occupy an independent species position. At the same time, the existing 6% difference between the sequences of the 16S rRNA genes of the new isolates, in addition to the above-mentioned phenotypic differences (Tables 1 and 2), prevents us from affiliation of these strains to the one and the same species. The neutrophilic freshwater species of the genus *Clostridium, C. felsineum* [17] and *C. formicoaceticum* [18], as well as the moderately haloalkaliphilic species of the genus *Natronincola, N. histidinovorans* [19], are the closest relatives of the new strains. The new isolates differ considerably from *C. felsineum* and *C. formicoaceticum* by obligate alkaliphily, resistance to NaCl, and ability to grow in the presence of carbonates, which evidently results from their fundamentally different habitats (Table 2). The newly isolated strains did not utilize carbohydrates, and they were specialized in peptide utilization. The species *N. histidinovorans* [19], *Tindallia californiensis* [20], and *T. magadiensis* [1], isolated previously from highly mineralized soda lakes, are phenotypically much closer to the new isolates. The genera *Natronincola* [19] and *Tindallia* [1] proposed by us were reclassified from the genus *Clostridium*, cluster XI, due to their phylogeny and phenotypic characteristics. They include obligately alkaliphilic but slightly [1] or moderately halophilic bacteria [19], and are acetogenic primary anaerobes. They do not utilize carbohydrates and utilize a few amino acids, peptone, and certain compounds of the central metabolism (pyruvate or citrate). The new isolates differ from the *Tindallia* species [1, 20] (88.5–90% 16S rRNA gene similarity) in morphology, inability to utilize the amino acids of the ornithine cycle and citrate, and in the lipid content and composition. *N. histidinovorans* [19] is phenotypically and phylogenetically closer to the new strains. Similarly to *N. histidinovorans*, strain Z-0511 was found to be capable of growth on histidine and weak growth on glutamate; strain Z-7031 was able to grow on pyruvate (Table 2). The new isolates are close to *N. histidinovorans* with respect to the fatty acid profiles. As in the case of *N. histidinovorans* [18], $C_{16:1\omega 7c}$, $C_{16:0}$, $C_{14:0}$, and $C_{18:0}$ were the major fatty acids. The content of the C_{16} aldehyde in the membranes of the new isolates was lower (20–24%) than in the membranes of the *N. histidinovorans* cells, and the content of 9-octadecenoic acid ($C_{18:1\omega 9}$) was by an order of magnitude higher (13.6–16.4%).

According to their physiological and phenotypic characteristics and habitat, the new strains are most close to members of the genus *Natronincola*. Although, according to the results of the comparative analysis of their 16S rRNA gene sequences, the species *C. felsineum, C. formicaceticum, N. histidinovorans*, and the new isolates form a single cluster, they cannot be classified into one genus due to the above-described phenotypic differences.

On the basis of the physiological, phenotypic, and phylogenetic differences between the strains Z-7031^T and Z-0511^T and their closest relatives from the cluster XI, we propose that these strains should be classified as new species of the currently monotypic genus *Natronincola, Natronincola ferrireducens* sp. nov. (Z-0511^T) and *Natronincola peptidovorans* sp. nov. (Z-7031^T).

Description of *Natronincola ferrireducens* sp. nov. (Zavarzina, Tourova, Osipov)

L. gen. n. *ferri*, iron; L. part. adj. *reducens*, to reduce; N. L. *ferrireducens*, an iron-reducing organism.

The cells are thin straight or slightly curved motile rods, 1.5–8 × 0.2–0.5 μm. The cells are spore-forming. The spores are terminal, and of a spherical shape. The cell wall structure is of the gram-positive type. Cell division occurs by septation. Obligate moderately aerotolerant catalase-positive anaerobe. Obligate alkaliphile; growth occurs in a pH range of 7.5–10.2, with an optimum at pH 8.4. Mesophilic; the temperature range for growth is 15– 40°C, with an optimum at 35–37°C. Halotolerant; growth occurs in a salinity range of NaCl 0–60 g/l, with an optimum at 5–7 g/l. Yeast extract, trypticase, tryptone, Bacto Soytone, meat extract, peptone, Casamino acids, histidine, and, to a lesser extent, glutamate are fermented. Carbohydrates, organic acids, and alcohols are not utilized. The Stickland reaction is not performed. Proteins are not hydrolyzed. Acetate, propionate, and formate (in trace amounts) are the main products formed in the course of fermentation of yeast extract. In the presence of yeast extract as a substrate, amorphous ferric hydroxide (AFH), EDTA-Fe(III), anthraquinone-2,6-disulfonate (quinone), Mn(IV), fumarate, and crotonate are reduced. AFH is reduced with the resulting formation of Fe(II) sesquioxides. The major fatty acids are $C_{16:1\omega 7c}$ (29.75%), $C_{16:0}$ (14.55%), $C_{18:1\omega 9}$ (16.40%), and $C_{14:0}$ (6.44%) $C_{18:0}$ (5.31%).

The DNA G+C content is 35.3 ± 0.5 mol%.

The isolation source is bottom sediments of soda lakes. The type strain was isolated from bottom sediment of the low-mineralization lake Khadyn (Tyva).

The type strain is Z-0511^T (VKM = B-2402, DSMZ 18346).

Description of *Natronincola peptidovorans* sp. nov. (Zhilina, Tourova, Osipov)

L. gen. n. *peptidum*, peptides; L. part. adj. *vorans*, devouring; N. L. *peptidovorans*, a peptide-devouring organism.

The cells are thin straight or slightly curved rods, 1.5–4.0–10 × 0.2–0.5 μm, actively motile by means of lateral flagella. The cells form terminal spherical spores. The cell wall structure is of the gram-positive type. The cells divide by septation. Obligate moderately aerotolerant catalase-positive anaerobe. Obligate alkaliphile, growing in a pH range of 7.5–10.2, with an optimum at pH 8.4–8.8. Mesophile; the temperature range for growth is 15– 40°C, with an optimum at 35–37°C. Halotolerant; growth occurs in a salinity range of up to 70 g/l NaCl, with an optimum at 10–30 g/l. Chemoorganoheterotrophic fermenter. Fermented are yeast extract, trypticase, tryptone, Bacto Soytone, meat extract, peptone, Casamino acids, and pyruvate. Carbohydrates, individual amino acids, organic acids, and alcohols are not utilized. The Stickland reaction is not performed. Proteins are not hydrolyzed. Acetate, formate (1 : 1), and trace amounts of H_2, propionate, and isobutyrate are the products formed in the course of fermentation of yeast extract. In the presence of yeast extract as a substrate, amorphous ferric hydroxide (AFH), anthraquinone-2,6-disulfonate (quinone), and Mn(IV) are reduced. AFH is reduced with the formation of Fe(II) sesquioxides and magnetite. The major fatty acids are $C_{16:1\omega7c}$ (25.11%), $C_{16:0}$ (15.37%), $C_{18:1\omega9}$ (13.58%), $C_{14:0}$ (11.97%), and $C_{15:0}$ (9.28%), $C_{18:0}$ (4.42%).

The DNA G + C content is 35.5 ± 0.3 mol%.

The isolation source is bottom sediments of soda lakes. The type strain was isolated from the bottom sediment of the low-mineralization Lake Verkhnee Beloe (Transbaikal Region).

The type strain is Z-7031^T (VKM = 2503, DSMZ 18979).

ACKNOWLEDGMENTS

We thank A.M. Lysenko for measuring the DNA G + C content, N.I. Chistyakova for solid-phase Mössbauer spectroscopy, and V.V. Kevbrin for help in determination of the metabolic products.

This work was supported by the program of the Presidium of the Russian Academy of Sciences "Origin of the Biosphere and Evolution of the Geobiologcal Systems" and the program NSh-1221.2008.4 (state contract no. 02.740.11.0023 with Russian Federal Agency of Science and Innovation (Rosnauka)).

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