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EXPERIMENTAL ARTICLES

Thermoacidophilic Microbial Community Oxidizing the Gold-Bearing Flotation Concentrate of a Pyrite-Arsenopyrite Ore

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Abstract—An aboriginal community of thermophilic acidophilic chemolithotrophic microorganisms (ACM) was isolated from a sample of pyrite gold-bearing flotation concentrate at 45–47°C and pH 1.8–2.0. Compared to an experimental thermoacidophilic microbial consortium formed in the course of cultivation in parallel bioreactors, it had lower rates of iron leaching and oxidation, while its rate of sulfur oxidation was higher. A new thermophilic acidophilic microbial community was obtained by mutual enrichment with the microorganisms from the experimental and aboriginal communities during the oxidation of sulfide ore flotation concentrate at 47°C. The dominant bacteria of this new ACM community were *Acidithiobacillus caldus* (the most active sulfur oxidize) and *Sulfobacillus thermotolerans* (active oxidizer of both iron and sulfur), while iron-oxidizing archaea of the family *Ferroplasmaceae* and heterotrophic bacteria *Alicyclobacillus tolerans* were the minor components. The new ACM community showed promise for leaching/oxidation of sulfides from flotation concentrate at high pulp density (S : L = 1 : 4).

Keywords: flotation concentrate of a gold-bearing sulfide ore, thermoacidophilic microbial communities, species composition of ACM communities

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Communities of acidophilic chemolithotrophic microorganisms (ACM) develop in the presence of sulfide minerals and products of their oxidation (elemental sulfur, reduced sulfur compounds, and ferrous iron). Such environments include sulfide ore deposits, mine water, pyritic coals, waste of mining and processing industry, and the pulp of reactors processing sulfide ore concentrates. Their activity results in acidification of the environment (sometimes to pH 0.5 and lower) and increased concentrations of heavy and toxic elements (copper, zinc, nickel, cobalt, arsenic, antimony, etc.). Such microorganisms are therefore considered extreme chemolithotrophic acidophiles. These organisms belong to archaea and bacteria (gram-positive and gram-negative). Interaction of microbial populations in communities includes competition for the substrates and their common utilization. This can be seen in the case of sequential decomposition of a complex substrate, such as sulfide minerals; in this process, every component produced by oxidation is utilized by certain organisms, and products of their metabolism or cell lysis are used by other organisms. ACM communities comprise chemolithoautotrophic, mixotrophic, and organoheterotrophic organisms [1]. Species composition is richest in mesophilic communities. At higher temperatures, the range of microorganisms forming communities becomes limited. At the temperatures exceeding 70°C, only archaea are involved in the processes of oxidation of inorganic substrates at low pH values.

Depending on the oxidized substrate and the temperature mode, different species predominate in the ACM communities involved in biogeotechnological processes. Thus, *Acidithiobacillus* (*Ac.*) *caldus* and *Leptospirillum ferriphilum* were the dominant species in the continuous process of cobalt- and iron-containing concentrate leaching [2]. Switching from leaching of a concentrate containing FeS, FeS_2, and FeAsS to leaching of the pyrite–arsenopyrite concentrate resulted in elimination of the minor species and changes in the ratio of the dominant ones [3, 4]. Samples of the solid and liquid phases from heap leaching of chalcocite yielded new species; except for *Ac. caldus* strains, a new archaeal species '*Ferroplasma cyprexacervatum*' was isolated [5]. The latter was subsequently reclassified as '*Ferroplasma cupricumulans*' [6] and then as *Acidiplasma cupricumulans* [7]. Being the best adapted to specific substrates and climatic conditions, aboriginal microorganisms forming communities in natural ecosystems exhibit the highest oxidation activity [8, 9].

The goal of the present work was to obtain a thermoacidophilic chemolithotrophic microbial community actively leaching the pyrite–arsenopyrite flota-

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tion concentrate and to characterize the species composition of this community.

MATERIALS AND METHODS

Subjects of Research

The subjects of research were gold-containing silica-alumine flotation concentrate of a refractory pyrite–arsenopyrite ore and communities of thermophilic acidophilic chemolithotrophic microorganisms (ACM).

Concentrate composition. The concentrate consisted of silica-alumina mass with inclusions of sulfide minerals. The size of flotation concentrate particles was –0.074 mm (pulverescent). Pyrite (35%) and arsenopyrite (8%) were the major sulfide minerals; small amounts of stibnite (0.56%) were present. Silicate and carbonate minerals constituted 49 and 5.5%, respectively. According to chemical analysis, flotation concentrate contained 20.9% total iron, 19.2% sulfide iron, 3.63% arsenic (total and sulfide one), 20.44% total sulfur, 19.87% sulfide sulfur, 0.08% elemental sulfur, 0.56% total antimony, 0.28% sulfide antimony, 1.42% calcium, and 1.58% magnesium. The analysis was carried out using a 3100 atomic adsorption spectrometer (Perkin Elmer, United States) with plasma atomization.

Aboriginal ACM community. The aboriginal acidothermophilic microbial community was isolated from flotation concentrate samples by increasing the pulp density (S : L) from 1 : 100 to 1 : 10 using the standard 9K medium without ferrous iron, pH 1.8–2.0, as the liquid phase [10]. The medium contained the following (g/L): $(NH_4)_2SO_4$, 3.0; KCl, 0.1; $K_2HPO_4 \cdot 3H_2O$– 0.5; $MgSO_4 \cdot 7H_2O$ – 0.5; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01, and yeast extract (YE), 0.02%. The flasks (250 mL) with 100 mL of the pulp were incubated on a shaker (190 rpm) at 45 or 47°C. Adaptation of the microbial community to increasing pulp density was carried out gradually, based on the values of the controlled parameters. H_2SO_4 (10 N) and $NaHCO_3$ (20%) were used to adjust pH of the pulp liquid phase to 2.0. At every stage of pulp densening, the process of biochemical oxidation was carried out until a practically stable ferric iron concentration was reached, acidification of the liquid phase stopped, and a considerable part of the cells attained a dormant state.

Experimental ACM community. The experimentally developed thermoacidophilic microbial consortium, which oxidized sterile concentrate at 47°C after adaptation to high pulp density, was used for comparison with the aboriginal microbial community. This consortium contained thermoacidophilic bacterial and archaeal species from the collection of the Laboratory of Chemolithotrophic Microorganisms, Winogradsky Institute of Microbiology, which were able to oxidize preferentially elemental sulfur (S^0), reduced sulfur compounds (RSC), Fe^{2+}, or sulfide minerals (MeS). Bacterial strains belonged to *Leptospirillum ferrooxidans* and *Sulfobacillus* species: *S. thermosulfidooxidans*, *S. thermosulfidooxidans* subsp. *asporogenes, S. sibiricus, S. thermotolerans*, and *"S. olympiadicus"*); archaea belonged to the species *Ferroplasma acidiphilum*.

Efficiency of bioleaching and oxidation of sulfide concentrate was investigated on a laboratory setup with two parallel reactors (2.5 L, 1 L of the medium, aeration 3 min^{-1}, and 47°C) for the experimental and aboriginal communities.

Since nonsterile ore concentrates are used in biotechnologies for noble metal recovery, aboriginal organisms developed within the experimental community (EC), with their abundance increasing with subsequent transfers. The communities were mutually enriched with the most active cultures most adapted to the oxidation substrate, and a new, combined ACM community was formed.

Methods of Research

The following parameters were monitored during the cultivation: pH, Eh, Fe^{3+} and Fe^{2+} concentrations, cell number, morphological diversity, and the dominant microorganisms. The ACM communities were grown in batch mode in flasks or in bioreactors using the medium described above.

Analysis of the pulp liquid phase. The analyzed parameters of the pulp liquid phase included: pH and Eh determined with a pH-150M meter (Belarus); concentrations of ferric and ferrous iron determined by chelatometric titration with Trilon B [11]; total arsenic determined by iodometric titration with iron ions bound with $TiCl_3$ [12]. Quantitative assessment of microbial cells was carried out by direct counts and by terminal tenfold dilutions. Microscopy was carried out using a Mikmed phase contrast microscope (LOMO, Russia) and an Olympus CX41 microscope (Japan). Total preparations and ultrathin sections were examined under a JE-100B (Jeol, Japan) electron microscope at 80 kV.

Isolation and identification of the cultures from a new combined thermophilic ACM community. Pure cultures of the strains comprising the new ACM community and oxidizing flotation concentrate in dense pulp at 47°C were isolated on selective media and by tenfold terminal dilutions in three replicas. The media used were 9K mineral base with yeast extract (YE) and the major oxidized substrate: sterile flotation concentrate (pH 2.0), 10 g/L ferrous iron (pH 1.8), or 10 g/L elemental sulfur (pH 2.3). To obtain monocultures, the spores found in the medium were heated at 100°C for 35 min and plated onto the media or inoculated in the media with organic (0.02–0.1%) and/or mineral energy substrates providing for spore germination and growth of acidophilic chemolithotrophic bacilli.

Purity of the cultures was determined by control plating on the medium with 0.5% agarose with subsequent transfer of the colonies into liquid medium; by transfers into the media with organic substrates in regular (0.02%) and high concentrations (10 g/L), inhibiting growth of chemolithotrophic mixotrophs; and by repeated transfers into 9K medium with Fe^{2+}/S^0 with or without YE.

Identification of the species of archaea and gram-positive and gram-negative bacteria involved in sulfide mineral oxidation as members of the thermophilic ACM community was carried out by both molecular biological and conventional cultural microbiological techniques for determination of their growth in media with mineral (1% S^0, 0.25% $K_2S_4O_6$, 1% MeS) and organic substrates (0.02%): carbohydrates, amino acids, YE, casamino acids, and casein hydrolysate.

To determine the 16S rRNA gene sequences, late-exponential phase cells were collected by centrifugation (10000 *g*, 10 min) and washed with the 9K salt base. DNA was extracted using the DNA-EKSPRESS kit (NPF Litekh, Russia) according to the manufacturer's recommendations. Concentrations of the DNA preparations were 30–50 μg/mL. Electrophoresis confirmed the presence of RNA in trace amounts (<1%).

Amplification of the 16S rRNA genes from pure bacterial cultures was carried out using the universal primer system: 27F (5'-AGAGTTTGATCMTGGCT CAG-3') and 1492R (5'-GGTTACCTTGTTAC GACTT-3') [13]. For identification of sulfobacilli (after inoculation in selective media), amplification was carried out using the primer pair 27F and Stherm-P1 (5'-GCTCACGAGCGTGTCCAGT-3'), which is specific for *Sulfobacillus* species—*S. thermosulfidooxidans, S. sibiricus, S. thermotolerans*, and *S. olympiadicus* [14]. The PCR profile was as follows: first cycle, 94°C, 5.5 min; 57°C, 45 s; 72°C, 1.5 min; 30 cycles, 94°C, 30 s; 57°C, 45 s; 72°C, 1.5 min; and final cycle, 72°C, 7 min.

Amplificates of the 16S rRNA gene fragments of the archaeon were obtained using the primer pair A23F (5'-TCCGGTTGATCCTGCC-3') specific for the family *Ferroplasmaceae* and 1492R. The PCR profile was as follows: first cycle, 98°C, 2 min; 96°C, 1 min; 45°C, 1 min; 72°C, 2 min; 30 cycles, 96°C, 1 min; 45°C, 1 min; 72°C, 2 min; and final cycle, 72°C, 10 min [7].

Amplification mixture (20 μL) contained the following: 1× Taq DNA polymerase buffer (17 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; and 2 mM $MgCl_2$); 2.5 nmol of each dNTP; 10–50 ng DNA template; 5 pmol of each primer; and 2 U Taq DNA polymerase (Evrogen, Russia). PCR was carried out on an MJ Mini Gradient Thermo Cycler (Bio-Rad, United States). PCR products were analyzed by electrophoresis in 1% agarose gel with ethidium bromide at 6 V/cm. The amplicons were isolated and purified using the Cleanup Mini reagent kit (Evrogen, Russia) according to the manufacturer's recommendations.

The PCR fragments of the 16S rRNA genes were sequenced according to Sanger et al. [14] using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, United States) on a 3730xl DNA Analyzer automatic sequencer (Applied Biosystems, United States) according to the manufacturer's recommendations. The primers listed above were used for sequencing, with reading carried out in two directions.

Preliminary analysis of the similarity of the 16S rRNA sequences was carried out using the NCBI BLAST server (http://www. ncbi.nlm.nih.gov/blast). The sequences were aligned with a representative selection of the 16S rRNA sequences of most closely related bacterial and archaeal species using the CLUSTALW software package (http://www.ebi.ac.uk/Tools/msa/clustalw2) [15]. The sequences of strains PCG-1 (1433 bp), PCG-2 (874 bp), PCG-3 (1407 bp), and PCG-4 (1349 bp) corresponded to *Escherichia coli* positions 38–1473, 51–930, 52–1447, and 53–1410. Phylogenetic trees were constructed using the algorithms implemented in the TREECONW software package [16]. Statistical significance of the branching order was determined by bootstrap analysis of 1000 alternative trees. The resulting 16S rRNA gene sequences were deposited to GenBank under accession nos. KF940049–KF940052.

Statistical treatment of the results was carried out using the Student's criterion at 5% significance level [17].

RESULTS AND DISCUSSION

Aboriginal ACM Community and Its Adaptation to High Pulp Density

Physiological and morphological characteristics. In the course of isolation of the aboriginal community responsible for leaching and oxidation of metal sulfides from ore flotation concentrate at 45 and 47°C, the cells were first observed on the third day (10^6/mL liquid phase of the medium). On the fourth day their number increased to 10^8/mL. The subsequent densening of the pulp to S : L = 1 : 10 resulted in cell numbers increasing to (1.5–2.0) × 10^9/mL. After ten days, pH of the liquid phase decreased to 1.6–1.7, the redox potential (Eh) increased to 820–834 mV, and the concentration of leached/oxidized iron at 45 and 47°C was 9.05 and 12.17 g/L, respectively.

On days 3–4, medium-sized rods with rounded ends 0.7 × (1.3–1.4) μm predominated in the community. These cells were found to be motile. Gram reaction was negative. Other morphotypes present in the community included longer, gram-positive rods similar to sulfobacilli in cell size and capacity for sporulation, and less common coccoid cells and small rods

Table 1. Growth parameters of the aboriginal microbial community adapted to increasing pulp density at two temperature values

Pulp density, g/100 mL	45°C				47°C			
	pH	Eh, mV	Fe^{3+}/Fe^{2+}, g/L	cell number/mL × 10^9	pH	Eh, mV	Fe^{3+}/Fe^{2+}, g/L	cell number/mL × 10^9
11	1.67	860	11.07/0.07	1.2	1.60	850	14.31/0	0.6
12	1.95	854	12.75/0	1.3	1.38	783	15.24/0.4	1.6
14	1.79	857	14.83/0.07	1.25	1.58	775	17.87/0	1.2
16	1.72	854	16.91/0	1.2	1.81	818	21.79/0	1.8
20	1.67	844	21.19/0.21	2.0	1.68	811	26.46/0	1.8

Iron concentration values include mass exchange.

with tapered ends. On days 8–10 cell numbers of these morphotypes increased, and larger rods, 0.8 × (1.5–2.5) μm capable of sporulation (presumable sulfobacilli or alicyclobacilli), as well as small rounded forms (presumable archaea) were revealed. Gram-negative bacteria described above remained, however, predominant. Control transfers confirmed the microscopic observations. The cells of the dominant gram-negative form grew under autotrophic conditions with sulfur, gram-positive bacteria preferred mixotrophic conditions and oxidized sulfur or iron in the presence of YE, while cocci grew in the medium with iron, tetrathionate, and YE.

Stability of the community. Stability of the ACM community at increasing pulp density was studied. Pulp densening was carried out at batch mode at 45 and 47°C. Adaptation to pulp density increase from 10 to 20 g/100 mL took 7 and 5 days at 45 and 47°C, respectively. Comparative parameters of the adaptation are listed in Table 1.

At pulp density of 20 g/100 mL, Fe^{2+} leaching and oxidation to Fe^{3+} were more pronounced at 47°C than at 45°C. Total concentrations of iron leached and oxidized by the aboriginal community at 45 and 47°C were 21.40 and 26.46 g/L, respectively, while the differences in cell number were insignificant. The aboriginal microbial community retained its structure. It was subsequently cultivated at 47°C, since substrate oxidation under these conditions was more active and rapid.

Investigation of resistance of this aboriginal community to higher pulp density in flasks (S : L = 1 : 5, 20 g/100 mL) revealed that after 5 days the total concentration of leached and oxidized iron remained at the same level, while pH decreased from 2.0 to 1.45, indicating active RSC oxidation. Arsenic concentration was 2.66 g/L. The structure of the aboriginal community was retained, while the number of microbial cells increased to 2.8×10^9 cells/mL.

Selection of the ACM community. The goal of this stage of investigation was to select the microbial community (aboriginal or experimentally formed) in order to achieve the highest rates of bioleaching and oxidation of flotation concentrate from refractory sulfide ores. The composition of the experimental ACM community, comprising various species of sulfobacilli, leptospirilli, and archaea, was described in Materials and Methods.

After successful gradual adaptation of the experimental consortium to high pulp density (S : L = 1 : 5) with sterile sulfide ore flotation concentrate, two communities—the experimental and the aboriginal ones—were compared. Cultivation in parallel bioreactors was carried out in batch mode. Inoculum was 10% (vol/vol). The results are listed in Table 2. It may be seen that after 4 days of growth at maximum pulp density the experimental community was more active in iron leaching/oxidation than the aboriginal one. The values of cell yield, Eh, and arsenic leaching by these communities (2.8 and 2.7 g/L) were almost similar. Active oxidation of sulfide sulfur (according to pH decrease) to sulfate (which is highly important at the last stage of the biotechnological process, during gold cyanidation) was, however, more significant in the variant with the aboriginal ACM community. Moreover, additional tests of stability of the structure of the experimental microbial community revealed that an increasing number of the cells characteristic of the aboriginal community was present in the experimental community at increasing pulp density (when nonsterile flotation concentrate was used) and extended duration of cultivation. This finding was confirmed by plating on selective media. These organisms were mainly gram-negative sulfur-oxidizing motile rods, 0.6 × (1.3–1.4) μm and constituted the dominant component of the aboriginal ACM community, which was most easily isolated from flotation concentrate samples. Archaea growing on tetrathionate, apart from iron, were observed, as well as very small forms, including motile ones. Iron-oxidizing leptospirilli and some sulfobacilli were no longer present in the experimental community. This new thermophilic ACM community retained the main characteristics of both communities (the experimental and aboriginal one) and exhibited high iron- and sul-

Table 2. Biooxidation of sulfide ore flotation concentrate by the experimental and aboriginal ACM communities in batch mode at 47°C and various pulp densities

Pulp density, g/L	Experimental community				Aboriginal community			
	pH	Eh, mV	Fe^{3+}/Fe^{2+}, g/L	cell number/mL × 10^9	pH	Eh, mV	Fe^{3+}/Fe^{2+}, g/L	cell number/mL × 10^9
70	1.73	802	5.18/1.26	1.3	1.40	841	7.06/0.7	1.7
90	1.56	871	13.33/0	2.25	1.41	865	14.39/0	2.5
100	1.48	880	20.79/1.54	2.8	1.41	808	16.93/0	3.2
120	1.45	780	28.18/0	2.75	1.32	862	20.84/0.41	2.75
140	1.43	823	31.28/0.5	3.0	1.59	855	21.67/0	3.0
160	1.38	859	31.33/1.57	3.5	1.50	860	23.30/0	2.0
180	1.48	820	35.48/1.12	2.5	1.40	856	30.7/0.28	2.5
200	1.54	853	37.59/0.98	3.5	1.40	836	32.1/0.7	2.5

Iron concentration values include mass exchange.

fur-oxidizing activity. The data presented on Fig. 1 demonstrate the stable activity of the new ACM community enriched with the active organisms from both communities, at elevated pulp density in the reactors. Pulp density is an important economical factor in biotechnology.

The new thermophilic ACM community was found to retain its activity at still higher pulp density (S : L = 1 : 4) under conditions of batch cultivation in a tank. In the course of this process, cell numbers were as high as 3.1×10^9/mL, Eh was 871 mV, and the concentrations of leached arsenic and oxidized iron were 4.36 and 43.21 g/L, respectively. RSC oxidation was accompanied by a considerable pH decrease to 0.89 (Fig. 1).

Isolation of Monocultures from Pulp Samples of the Thermophilic ACM Community

Isolation and identification of bacteria. The major members of the new thermophilic ACM community enriched with the active organisms from both the aboriginal and the experimental communities were isolated from the samples of the liquid phase of high-density pulp (see Materials and Methods).

Strain PCG-1. A rod-shaped gram-negative bacterium, strain PCG-1, growing under chemolithoautotrophic conditions with elemental sulfur or tetrathionate, was the dominant species (35–60%) isolated from most samples. Its cell number varied from 10^7 to 10^9/mL, depending on conditions. The cells were monotrichous with a polar flagellum (Fig. 2). Cell size and morphology depended on the medium composition and cultivation time. The cells were rods with round ends, often in pairs, sometimes of coccoid shape; their size varied within the range of (0.4–0.6) × (0.6–2.0) μm. Phase contrast microscopy revealed motility of cell pairs. Electron microscopy confirmed the typical gram-negative structure of the cell wall (Figs. 2a–2e) and its undulated shape. The cells were covered with a mucous microcapsule (Fig. 2a). The mucus provided for cell aggregation (Fig. 2b) and sorption at the surface of the sulfur crystals. The cytoplasm was relatively dense, with polyhedral ((90–120) × (110–200) nm) and rounded (130 × 160 nm) inclusions in the center and periphery. These are probably carboxysomes containing ribulosebisphosphate carboxylase, the enzyme responsible for CO_2 fixation, similar to those revealed in the cytoplasm of *Acidithiobacillus thiooxidans* cells [18]. The cells of strain PCG-1 contained also the granules, presumably of polyhydroxyalkanoates (PHB) and polyphosphates. Various degrees of complexity of organization of the cell wall, membranous apparatus, and cytoplasmic inclusions have been reported for various

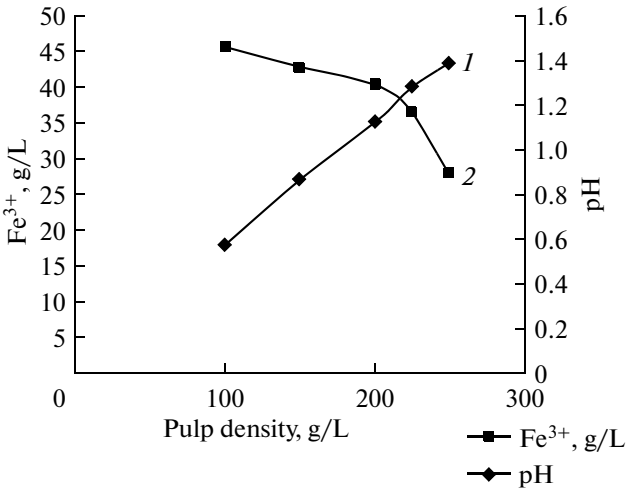

Fig. 1. Fe^{3+} concentration and oxidation of reduced sulfur compounds (RSC) depending on pulp density in the course of growth of the new ACM community: iron concentration, g/l (*1*) and final pH of the pulp liquid phase (*2*).

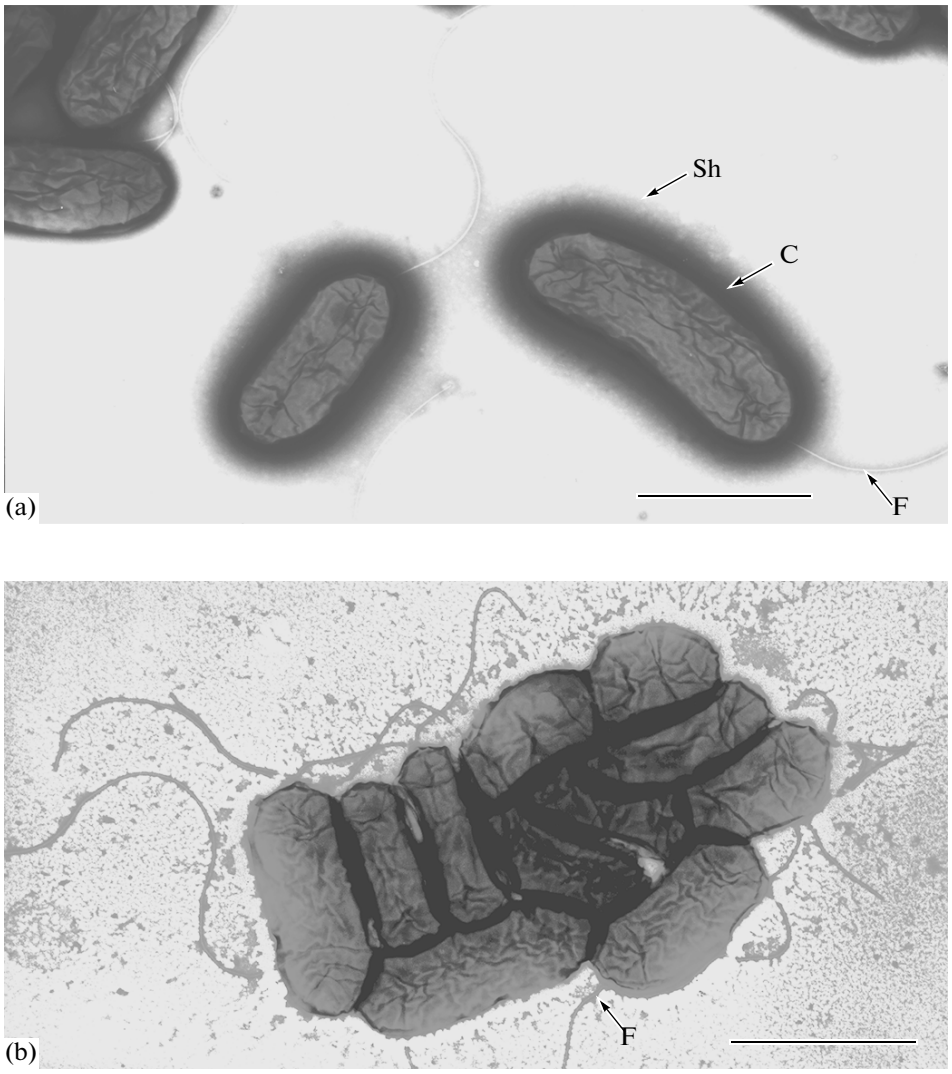

Fig. 2. Electron micrographs of the cells of the strain *Ac. caldus* PCG-1. Total preparations (a, b) and ultrathin sections (c–e). Folded envelope, mucous sheath (Sh), capsule (C), and flagellum (F) (a). Aggregated cells, some retaining their flagella (b). Three-layered cell envelope, folded cell wall (CW), outer membrane (OM), periplasm (P), cytoplasmic membrane (CM), nucleoid (N), and intracellular inclusions: carboxysomes (CS) and granules of polyphosphates (PP) and poly-β-hydroxybutyrate (PHB) (c–e). Scale bar is 1 μm (a–c) and 0.5 μm (d–e).

strains of mesophilic sulfur-oxidizing bacteria *Ac. thiooxidans* [19]. Cell ultrastructure of the strains of thermophilic sulfur-oxidizing bacteria *Ac. caldus* has not been described.

The isolate PCG-1 grew under autotrophic conditions in the medium with sulfur or tetrathionate within the temperature range from 25 to 55 ± 1°C, with the maximal specific growth rates of 0.15 and 0.08 h^{-1}, respectively, at the optimal temperature of 47.5 ± 1°C It differed from the type strain *Ac.* (previously *Thiobacillus*) *caldus* DSM 8584^T by broader temperature range and a different temperature optimum (32–52 and 45 ± 1°C, respectively, for the type strain). At 47.5°C the new strain grew at pH 0.7–4.0 (compared to pH 1.0–3.5 for the type strain) with the optimum at pH 2.0–2.5, similar to that of the type strain. At pH 4.0 the cells were small coccoids. PCG-1 cells grew at the lowest pH values as long septated filaments, which was an indication of impaired synthesis and/or functioning of autolysins, resulting in long chains of unseparated cells developing at pH 0.7–1.0. On solid medium with YE and tetrathionate, the isolate formed small round, smooth, transparent colonies with a yellowish coloration in the center due to sulfur precipitation.

Strain PCG-1 was found to be unable to oxidize iron, while thiosulfate (added in portions of 0.25 mM/h in order to minimize its chemical degradation at pH 2.5) could act as a substrate for lithotrophic growth, as well as sulfur and tetrathionate.

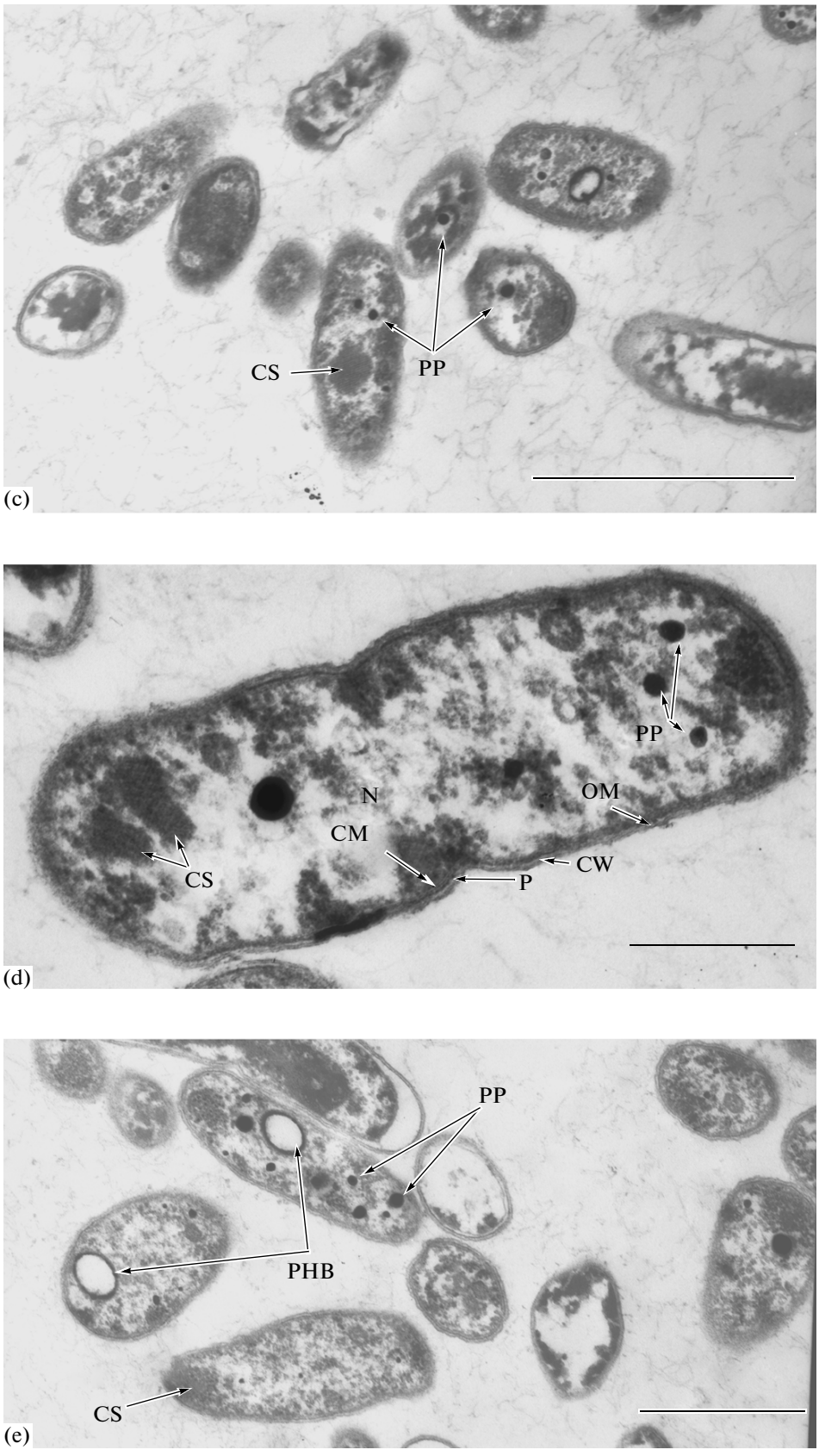

Fig. 2. Contd.

Sulfate (106.8 and 68.9 mM, respectively) was the terminal product of oxidation of RSC (S^0 and $S_4O_6^{2-}$); in the course of this process, pH decreased to 0.88 and 1.29 U, respectively. Similar to the type strain KU^T = DSM 8584^T [20], the biomass yield was 3–4 times higher in the presence of YE or glucose (0.02–0.03%), or glucose with YE in the medium with RSC, which was an indication of mixotrophic metabolism. Among the tested organic compounds (individual sugars, amino acids, casamino acids, and glycogen), casamino acids with or without glucose were found to support organoheterotrophic growth for 1–2 transfers. Cultivation of strain PCG-1 together with the type strain of *F. acidiphilum* Y-1 (from the collection of the Laboratory of Chemolithotrophic Microorganisms, Winogradsky Institute of Microbiology) resulted in joint pyrite oxidation.

Sequencing of the 16S rRNA gene fragment (1438 bp) of PCG-1 and comparison of this sequence (KF940049) with the GenBank sequences revealed the following. *Ac. caldus* strains most closely related to strain PCG-1 were DX-2 (DQ470072), N39-30-02 (EU49992), SM-1 (NR_102970), KU of *Ac.* (*Thiobacillus*) *caldus* type strain (Z29975 DSM 8584^T = ATCC 51766^T), and MBC-1 (C432648). The similarity between the 16S rRNA gene sequences of the isolate PCG-1 and strain DX-2; strains N39-30-02, SM-1, and the type strain *Ac. caldus* KU^T; and strain MBC-1 was 99.6, 99.4, and 98.5%, respectively. High similarity of the 16S rRNA gene sequences made it possible to identify the isolate PCG-1 as an *Ac. caldus* strain [21]. All the 16S rRNA gene sequences formed a common cluster together with the sequence of the type strain KU^T *Ac. caldus* KU^T (Fig. 3). *Ac. caldus* PCG-1 is beyond doubt an aboriginal strain of the ACM community. The experimental consortium did not contain this organism.

Strain PCG-2. A group of gram-positive bacteria resembling *Sulfobacillus* in their morphology, life cycle, and ability to grow in media with ferrous iron, sulfur, or sulfide minerals (pyrite, arsenopyrite, and stibnite) at 50–55°C constituted 30–40% of the total cell number in the ACM community oxidizing sulfide flotation ore concentrate. This bacterial group predominated when the community was grown at 48–50°C, as well in the growth dynamics of the ACM community.

Transfers of the new thermophilic ACM community oxidizing sulfide flotation concentrate onto selective media with Fe^{2+} under optimal temperatures resulted in predomination of the cells of the spore-forming bacterial strain PCG-2 with specific growth rate of 0.3–0.4 h^{-1}. Apart from iron, the isolate actively oxidized sulfur and the experimental sulfide ore concentrate. The cells were rod-shaped, with the size varying within the range of (0.7–0.9) × (1.2–2.3) μm, depending on the energy substrate and growth phase. The spores were subterminal or terminal, usually oval. The temperature and pH growth ranges for strain PCG-2 were 18–55°C and 1.0–5.0 pH U, respectively. The strain was able to grow for several transfers with some organic compounds (glutamate, reduced glutathione, glucose, xylose, sucrose, yeast extract, caseine hydrolysate, tryptone, soluble starch, and glycogen). The organism was mixotrophic. The combination of the physiological pro-perties of strain PCG-2 agreed with that of bacteria of the genus *Sulfobacillus*. Sequencing of amplicon of the fragment (874 bp) of the 16S rRNA gene of strain PCG-2 (KF940050) revealed the most closely related *S. thermotolerans* strains: strain Kuch-1 (JX966410), strain Kr1 of the type species (DQ124681), strain TH (JF510470), and strain RIV14 (AY007664). The similarity between the sequences of the isolate and strain Kuch-1 was 100%; that between the isolate and strains Kr1^T, TH, and RIV14 was 99.9%. High similarity between the 16S rRNA gene sequences made it possible to classify the isolate PCG-2 as a *S. thermotolerans* strain. All 16S rRNA gene sequences formed a single cluster together with the sequence of the type strain *S. thermotolerans* Kr1 = DSM 17362 (Fig. 4). The strain *S. thermotolerans* PCG-2, exhibiting 100% homology with the 16S rRNA gene sequence of strain Kuch-1, may be considered a strain originating from the experimental consortium, which became a part of the new ACM community.

Strain PCG-3. A heterotrophic bacillus physiologically similar to the strain K1 *Al. tolerans* [22] was the third identified strain from the thermoacidophilic microbial community (a minor component). The isolate grew well on media with a number of organic compounds and was capable of weak oxidation of sulfur or iron in 9K medium with yeast extract. The highest abundance of strain PCG-3 in the ACM community (3–5% of the total cell number) was found at the end of sulfide concentrate oxidation, when lysis of some of the cells in the community occurred.

Sequencing of amplicon of the fragment (1408 bp) of the 16S rRNA gene of strain PCG-3 and its analysis (KF940051) revealed the most closely related strains: *Alicyclobacillus* sp. AP-AC (HQ728274), *Alicyclobacillus* sp. OL-10-05, *Al. tolerans* DSM 16297 (AB222265), *Al. tolerans* K1 (NR_036984), and a gram-positive heterotrophic acidophilic bacterium Y004 (AY140236) with 100, 99.9, 99.8, 99.4, and 98.8% similarity, respectively. These data suggested that the strain related to *Alicyclobacillus* sp. AP-AC and to *Al. tolerans* strains was a component of the aboriginal community within the new combined ACM community.

Isolation and identification of archaea. Two archaea isolated from the new combined ACM community oxidizing sulfide ore flotation concentrate were isolated and identified as members of the family *Ferroplasmaceae* based on their ability to oxidize iron at 45°C.

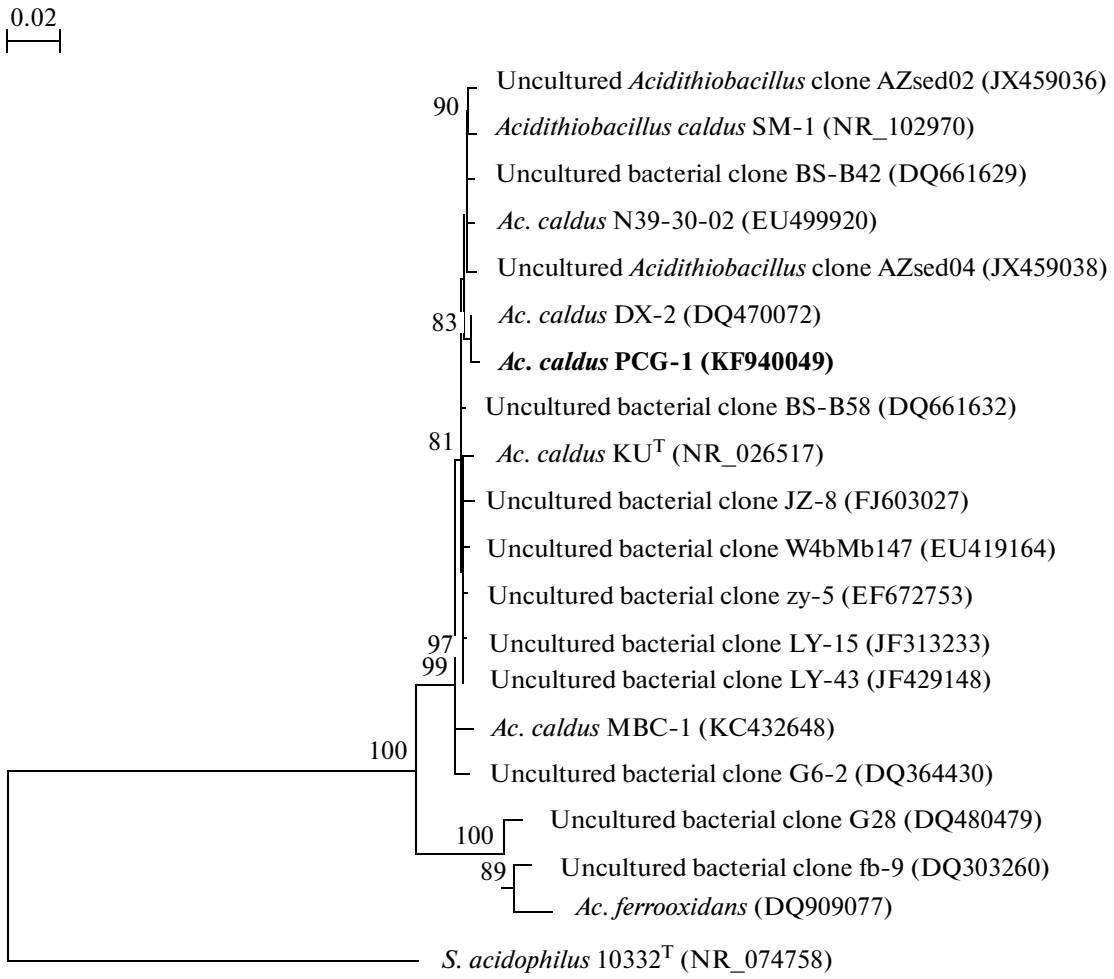

Fig. 3. Phylogenetic tree showing the position of the dominant strain *Ac. caldus* PCG-1 isolated from the new thermophilic ACM community. The tree was constructed using the neighbor-joining algorithm using the 16S rRNA gene nucleotide sequences of *Acidithiobacillus* strains. The scale shows the evolutionary distance corresponding to 2 replacements per 100 nucleotides. The numerals represent statistical reliability of the branching order determined by bootstrap analysis of 1000 alternative trees (>80%). The 16S rRNA gene sequence of *S. acidophilus* 10332^T (NR_074758) was used as an outgroup.

Strains PCG-4 and PCG-5. One of the strains occurred in the pulp liquid phase throughout almost all the stages of sulfide concentrate leaching/oxidation, while the highest numbers of the other one were observed at the last stage of the process in the tank with pulp with S : L = 1 : 4 and acidification to pH 1.3-1.4. The first archaeon, strain PCG-4, was a chemolithomixotroph and actively oxidized Fe^{2+} in the presence of YE (a required medium component for this organism). The second archaeon, strain PCG-5, did not possess such oxidative activity. It was a mixotroph requiring not only organic matter (YE), but also another inorganic substrate apart from iron: tetrathionate. The strain grew within a broad temperature range from 20 to 60°C. In this respect, as well as in morphology, strain PCG-5 was similar to the archaeon *Acidiplasma* (*Ferroplasma*) *cupricumulans* [5–7].

The fragment (1349 bp) of the 16S rRNA gene of the dominant archaeon, strain PCG-4, was sequenced (KF940052). Comparison with the GenBank sequences revealed that those of *Ferroplasma acidiphilum* strains were the most closely related: Kuch-3 (JX966412), OL 12-4 (KF 356026), and DR1 (AY 222042) exhibited ~100% similarity, while homology to the type strain *F. acidiphilum* Y^T (AJ224936) was 99.85%. Strain PCG-4, which certainly belongs to *F. acidiphilum*, has a broader temperature range for growth (15–50°C) than the type strain Y^T (15–45°C) and the temperature optimum at 45, rather than at o 35°C. It is probably more closely related to strains Kuch 3 or OL 12-4, which together with strain Y^T were a part of the experimental microbial consortium and had higher temperature resistance.

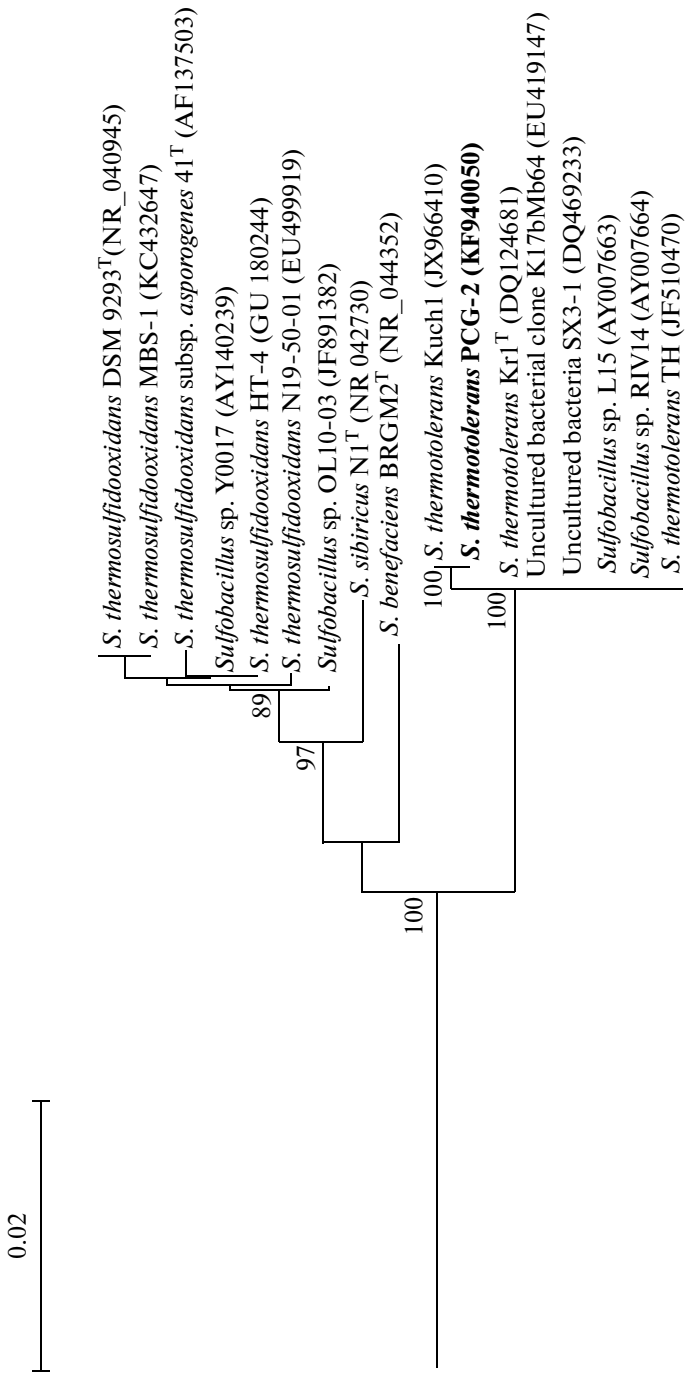

Fig. 4. Phylogenetic tree showing the position of the dominant strain *S. thermotolerans* PCG-2 isolated from the new thermophilic ACM community. The tree was constructed using the neighbor-joining algorithm using the 16S rRNA gene nucleotide sequences of *Sulfobacillus* strains. The scale shows the evolutionary distance corresponding to 2 replacements per 100 nucleotides. The numerals represent statistical reliability of the branching order determined by bootstrap analysis of 1000 alternative trees (>80%). The 16S rRNA gene sequence of *S. acidophilus* 10332^T (NR_074758) was used as an outgroup.

Thus, the dominant microorganisms of the new thermophilic ACM community exhibiting high rates of oxidation of sulfide forms of iron and arsenic, as well as of elemental sulfur produced in the course of oxidation of sulfide minerals, were aboriginal bacterial strains *Ac. caldus* PCG-1 and *Al. tolerans* PCG-3 (phylogenetically related to *Alicyclobacillus* sp. AP-AC and to *Al. tolerans* DSM 16297^T), an archaeon PCG-5, and

microbial strains from the experimental consortium: *S. thermotolerans* PCG-2 (phylogenetically related to *S. thermotolerans* Kuch-1) and the archaeon *F. acidiphilum* PCG-4 (phylogenetically related to strains Kuch-3 and OL 12-4). Stability of the composition of this community was subsequently confirmed by bio-oxidation of refractory pyrite–arsenopyrite gold-bearing flotation concentrate in continuous mode [23].

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