ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 6, pp. 842–849. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © A.G. Bulaev, T.A. Pivovarova, V.S. Melamud, B.K. Bumazhkin, E.O. Patutina, T.V. Kolganova, B.B. Kuznetsov, T.F. Kondrat'eva, 2011, published in Mikrobiologiya, 2011, Vol. 80, No. 6, pp. 854–861.

EXPERIMENTAL ARTICLES

Species Composition of the Association of Acidophilic Chemolithotrophic Microorganisms Participating in the Oxidation of Gold-Arsenic Ore Concentrate

A. G. Bulaev[a], T. A. Pivovarova[a], V. S. Melamud[a], B. K. Bumazhkin[b], E. O. Patutina[b], T. V. Kolganova[b], B. B. Kuznetsov[b], and T. F. Kondrat'eva[a, 1]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
[b] *Bioingeneering Center, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 1, Moscow, 117312 Russia*

Received February 2, 2011

Abstract—The species composition of the microbial association involved in industrial tank biooxidation of the concentrate of refractory pyrrhotite-containing pyrite-arsenopyrite gold–arsenic ore of the Olympiadinskoe deposit at 39°C was studied by cultural and molecular biological techniques. Pure microbial cultures were isolated, their physiological characteristics were investigated, and their taxonomic position was determined by 16S rRNA gene sequencing. The library of 16S rRNA gene clones obtained from the total DNA isolated from the biomass of the pulp of industrial reactors was analyzed. The diversity of microorganisms revealed by cultural techniques in the association of acidophilic chemolithotrophs (*Acidithiobacillus ferrooxidans, Leptospirillum ferriphilum, Sulfobacillus thermosulfidooxidans, Ferroplasma acidiphilum, Alicyclobacillus tolerans*, and *Acidiphilium cryptum*) was higher than the diversity of the 16S rDNA clone library (*At. ferrooxidans, L. ferriphilum*, and *F. acidiphilum*). The combination of microbiological and molecular biological techniques for the investigation of the biodiversity in natural and anthropogenic microbial communities promotes detection of new phylogenetic microbial groups in these communities.

Keywords: tank biooxidation, gold–arsenic concentrate, acidophilic chemolithotrophic microbial association, *Acidithiobacillus, Leptospirillum, Sulfobacillus, Ferroplasma*, 16S rRNA gene sequencing, 16S rRNA gene clone library.

DOI: 10.1134/S0026261711060038

Tank biooxidation of sulfide ore concentrates is presently used worldwide for recovery of precious metals [1]. The oxidation of sulfide ores and their concentrates is carried out by acidophilic chemolithotrophs, a physiologically heterogeneous microbial group comprising of the members of several bacterial and archaeal phyla. The possible practical application of these microorganisms results in interest in various aspects of their physiology, ecology, and phylogenetics [2]. Better understanding of the patterns of formation of associations of acidophilic chemolithotrophic microorganisms (ACM) in anthropogenic environments improves our knowledge of this unique physiological group of microorganisms. Intensification of the processes of recovery of precious and nonferrous metals and enhancing its efficiency require the determination of optimal conditions and technological regimes for the ACM-mediated oxidation of sulfide minerals [3].

The recent interest in microbial communities with the optimal growth temperatures of 40–60°C is due to the self-heating that occurs during tank biooxidation of sulfide minerals as a result of exothermic reactions [4]. Temperature has a significant effect on the species composition of the ACM populations. While *Leptospirillum ferrooxidans, L. ferriphilum*, and *Acidithiobacillus caldus* predominate in tank bioleaching at 40–45°C, when *At. ferrooxidans, At. thiooxidans*, and *Sulfobacillus* sp. are also present [4–7], *Acidithiobacillus caldus, Sulfobacillus* sp., and *Acidimicrobium* sp. prevail at 45–55°C [8,9].

We concentrated on the study of the microbial population which oxidizes sulfide minerals of the pyrrhotite-containing pyrite–arsenopyrite ore concentrate from the Olympiadinskoe deposit during gold extraction [10]. Previously, oxidation of the sulfide minerals of the flotation concentrate in the semi-industrial setup of the Polyus gold-recovery factory (GRF) was carried out at 30–35°C. Under these conditions, mesophilic gram-negative bacteria *At. ferrooxidans* and *At. thiooxidans* predominated in the microbial community. Scaling of the process resulted in a temperature increase caused by self-heating of the pulp. The temperature was maintained at 39–40°C by cooling the reactors with water. Analysis of the microbial association revealed the dominance of *Sulfobacillus* species. The new species *'S. olympiadicus'* was isolated.

[1] Corresponding author; e-mail: kondr@inmi.host.ru

Several years of monitoring the microbial association in the reactor pulp demonstrated that some *Sulfobacillus* strains were replaced by others, the strains of *S. thermotolerans* and *S. thermosulfidooxidans* were isolated. The strains of *L. ferrooxidans, Ferroplasma acidiphilum, At. ferrooxidans, At. thiooxidans,* and of the fungus *Aspergillus niger* were also present [11, 12].

Investigation of strain polymorphism in ACM revealed that phylogenetically related strains sometimes differed significantly in their physiological characteristics, affecting the role of specific isolates in the bioleaching processes [13, 14].

The goal of the present work was to apply molecular biological and cultural techniques to investigate the species composition of the association of acidophilic chemolithotrophic microorganisms involved in industrial tank biooxidation of the ore concentrate from the Olympiadinskoe deposit at 39°C, the physiology of pure cultures, and the role of each member of the association in the oxidation of sulfide minerals.

MATERIALS AND METHODS

Subjects of investigation. The microbial community of a sample from the Polyus CRF reactor was investigated. The sulfide ore concentrate contained the following (wt %): Fe_{total}, 25.6; As_{total}, 9.39; Sb_{total}, 9.50; S_{total}, 22.2, Ca, 3.00; and C, 1.37.

Isolation of pure cultures. Pure cultures were obtained by inoculation of selective media with terminal tenfold dilutions of the liquid phase of the pulp at 30, 35, 40, and 50°C. The strains of *At. ferrooxidans* were isolated on 9K medium [15] and the strains of *At. thiooxidans* and *At. caldus*, in the medium of the same mineral composition with iron sulfate replaced by elemental sulfur (10 g/l) as an energy source. *Sulfobacillus* sp. strains were isolated on 9K medium supplemented with yeast extract (YE, 0.02%) [16]. *Leptospirillum* sp. strains were isolated in the medium [7] containing ferrous iron (500 mM). *Ferroplasma* sp. was isolated in a modified 9K medium [17]. The microorganisms were incubated on a rotary shaker (170 rpm) in 250-ml Erlenmeyer flasks with 100 ml of the medium and 10 ml of the inoculum.

Phenotypic characterization of the microorganisms. The microorganisms were enumerated by direct count under an Amplival phase contrast microscope (Carl Zeiss, Germany). The effect of temperature on microbial growth was determined in a 9K medium. The capacity for oxidation of sulfur and ferrous iron under autotrophic and mixotrophic conditions was determined. The concentrations of ferric and ferrous iron ions were determined by complexometric titration [18]. Sulfur oxidation was assessed as a decrease in pH of the medium.

Genetic Systematic Investigation

DNA isolation from bacterial biomass was carried out as described in [19]. The DNA concentration in the preparations was 30–50 μg/ml. RNA was present in the preparations in trace amounts (less than 1%; the data of electrophoretic analysis are not shown).

Amplification of the 16S rRNA genes and cloning and sequencing of the PCR products. Polymerase chain reaction, cloning of the 16S rRNA gene PCR fragments, and sequencing of the clonal inserts of eubacterial origin were carried out with the universal primers [20]. For PCR and subsequent sequencing of the archaeal component, the original primer system was used [21]. In both cases, the amplification mixture (50 μl) contained the following: 1 ×BioTaq DNA polymerase buffer (17 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, and 2 mM $MgCl_2$), 12.5 nmol of each dNTP, 50 ng of template DNA, 5 pmol of the relevant primers, and 3 U of BioTaq DNA polymerase (Dialat Ltd., Russia).

PCR was carried out on a Gradient MasterCycler DNA amplifier (Eppendorf, Germany) according to the following profile: first cycle (9 min at 94°C, 1 min at 55°C, and 2 min at 72°C); and the final cycle of 7 min at 72°C.

The PCR products were analyzed by electrophoresis in 2% agarose gel at 6 V/cm.

The PCR products were recovered from low-melt agarose and purified using the Wizard PCR Preps reagent kit (Promega, United States) according to the manufacturer's recommendations.

Amplification products were cloned using the pGEM-T System reagent kit (Promega, United States) according to the manufacturer's recommendations.

Sequencing was carried out according to Sanger [22] using the Big Dye Terminator kit v.3.1 (Applied Biosystems, United States) on an ABI PRIZM 3730 automated sequencer (Applied Biosystems, United States) according to the manufacturer's recommendations. Both external and internal primers were used, and the reading was performed in two directions.

Analysis of the nucleotide sequences of the 16S rRNA genes. For the primary analysis of homologies of the 16S rRNA gene sequences, the BLAST server was used (http://www.ncbi.nlm.nih.gov/blast). These sequences were aligned with the relevant sequences of the closely related species using the CLUSTALW software package [23]. Phylogenetic trees were constructed using the procedures implemented in the TREECONW software package [24]. Statistical reliability of the resulting trees was determined by bootstrap analysis of 1000 alternative trees.

The results of 16S rRNA gene sequencing were deposited to GenBank under accession nos. JF891382–JF891387.

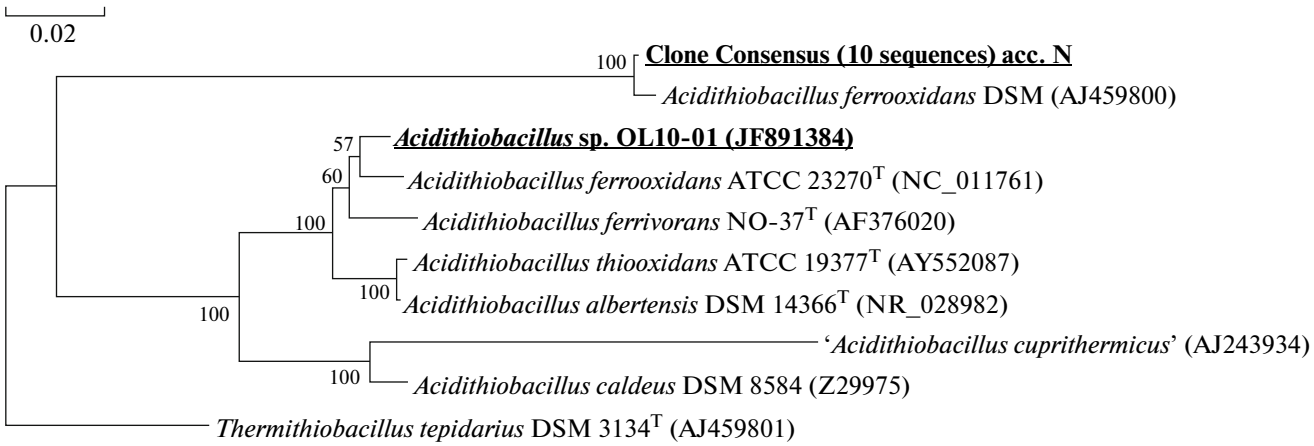

Fig. 1. Phylogenetic tree of the 16S rRNA gene sequences of the genus *Acidithiobacillus* showing the position of the strain *Acidithiobacillus* sp. OL10-01 and of the consensus phylotype. The numerals represent bootstrap values. The bootstrap values exceeding 70% are shown. The sequences obtained in the present work are marked by boldface. The evolutionary distance scale is shown at the top. The neighbor-joining algorithm was used. Type strains are designated by *T*. The 16S rRNA sequence of *Thermithiobacillus tepidarius* DSM 3134^T (GenBank AJ459801) was used as an outgroup.

RESULTS AND DISCUSSION

Isolation of Pure Microbial Cultures and Determination of Their Phylogenetic Position

Inoculation of terminal tenfold dilutions of the liquid pulp from the Olympiadinskoe deposit gold–arsenic ore resulted in the isolation of six microbial cultures. According to their morphological and physiological characteristics, the strains were tentatively classified as members of the genera *Acidithiobacillus, Leptospirillum, Sulfobacillus, Ferroplasma, Alicyclobacillus*, and *Acidiphilium*.

Comparison of the PCR fragments of the 16S rRNA genes (1300–1490 bp) with the similar sequences of various ACM species from GenBank [http://www.ncbi.nlm.nih.gov/nuccore] was used to determine the phylogenetic position of these strains. The 16S rRNA gene of strain *Acidithiobacillus* sp. OL10-01 (JF891384) was most closely related (98.5% similarity) to that of *At. ferrooxidans* ATCC 23270 (NC_011761) (Fig. 1). The sequence of *Leptospirillum* sp. OL10-02 (JF891387) exhibited 99.6% similarity to that of *L. ferriphilum* BY (EF025341) (Fig. 2). Strain *Sulfobacillus* sp. OL10-03 (JF891382) belonged to *S. thermosulfidooxidans* strain G2 (AY140233) (99.8% similarity), *Ferroplasma* sp. OL10-04 (JF891386) belonged to *F. acidiphilum* strain DR1 (AY222042) (98.4% similarity), *Alicyclobacillus* sp. OL10-05 (JF891385) belonged to *Al. tolerans* strain K1 (Z21979) (98.4% similarity), and *Acidiphilium* sp. OL10-06 (JF891383) belonged to *Ac. cryptum* strain ATCC 33463 (99.9% similarity).

Physiological Properties of the Strains

The bacterium *At. ferrooxidans* OL10-01 oxidized ferrous iron under autotrophic conditions (table). The optimal growth temperature was 35–37°C with the upper growth limit on medium 9K not exceeding 40°C (Fig. 3). The strain oxidized also elemental sulfur. The dynamics of sulfur oxidation by this strain at 35°C is shown in Fig. 4. The highest cell number (2.1×10^7/ml) was observed on the eighth day of growth, while pH decreased from 2.25 to 0.75. On the ninth day, the cell number decreased, while pH did not change. Strain OL10-01 (gF891384) did not oxidize sulfur at 40°C.

The bacterium *L. ferriphilum* OL10-02 oxidized ferrous iron under autotrophic conditions (table). The optimal growth temperature was 35–37°C, with the upper limit for growth at 45°C (Fig. 3). While the specific growth rates of *At. ferrooxidans* OL10-01 (JF891384) and *L. ferriphilum* OL10-02 (JF891387) during exponential growth at the optimal temperature were close (0.07 and 0.076 h^{-1}, respectively), the time required for complete oxidation of iron differed significantly (30 and 100 h, respectively). This is due to the long (3.5–4 days) lag phase of the leptospirilla.

The bacterium *S. thermosulfidooxidans* OL10-03 oxidized ferrous iron (Fig. 3) and sulfur at the optimal growth temperature of 45–50°C. The strain was capable of high rates of iron oxidation under mixotrophic conditions, although the rate of iron oxidation decreased sharply at elevated Fe^{3+} concentrations and the culture entered the stationary growth phase at 2.5 g/l ferrous iron, similar to the type strain of *S. thermosulfidooxidans* [16]. Under autotrophic conditions, iron was oxidized at a low rate. Sulfur was oxidized only under mixotrophic conditions, with the rate of sulfur oxidation depending on the YE concentration in the medium (Fig. 5). At 0.02% yeast extract, the number of cells decreased sharply (up to 5.7×10^7/ml) during the first day of incubation, while pH decreased

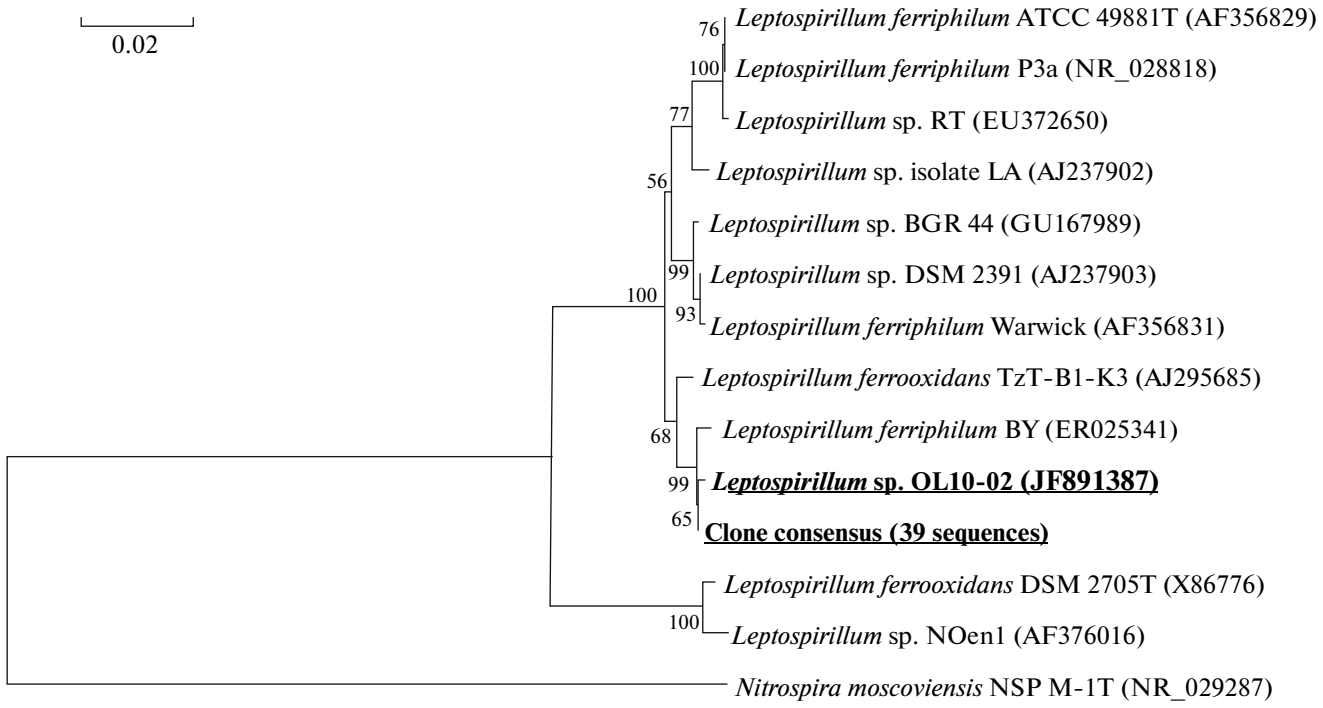

Fig. 2. Phylogenetic tree of the 16S rRNA gene sequences of the genus *Leptospirillum* showing the position of the strain *Leptospirillum* sp. OL10-02 and of the consensus phylotype. The numerals represent bootstrap values. The investigated sequences are marked by boldface. The evolutionary distance scale is shown at the top. The neighbor-joining algorithm was used. Type strains are designated by *T*. The 16S rRNA sequence of *Nitrospira moscoviensis* NSP M-1^T (GenBank NR_029287) was used as an outgroup. The sequences obtained in the present work are marked by underlined boldface.

from 2.25 to 1.5 of the second day. In the presence of 0.002% YE, growth commenced only after a two-day lag period and the yield was 1.9×10^7/ml. Since sulfobacilli are able to use yeast extract both for constructive metabolism and as an energy source and electron donor, its concentration in the medium is important.

The archaeon *F. acidiphilum* OL10-04 oxidized iron under mixotrophic conditions within the 30–45°C range with an optimum at 35°C.

The bacterium *Al. tolerans* OL10-05 oxidized elemental sulfur in the presence of 0.02% YE during the first two transfers (table). After five days of cultivation at

Diagnostic characteristics of the microorganisms isolated from the pulp of the Olympiadinskii GRF

Strain	Morphology	Isolation temperature, °C	Isolation medium	Substrates utilized
Acidithiobacillus ferrooxidans OL10-01 (JF891384)	Rods	30	9K	Ferrous iron, S^0
Leptospirillum ferriphillum OL10-02 (JF891387)	Vibrios	35	[10]	Ferrous iron
Sulfobacillus thermosulfido-oxidans OL10-03 (JF891382)	Rods	45	9KS	Ferrous iron, S^0 under mixotrophic conditions, YE
Ferroplasma acidiphilum OL10-04 (JF891386)	Pleomorphic cells	35	9K [17]	Ferrous iron under mixotrophic conditions
Alicyclobacillus tolerans OL10-05 (JF891385)	Spore-forming rods	40	Salt base of 9K	S^0 under mixotrophic conditions, YE
Acidiphilium cryptum OL10-06 (JF891383)	Cocci, diplococci	40	Salt base of 9K	YE*

* YE, yeast extract.

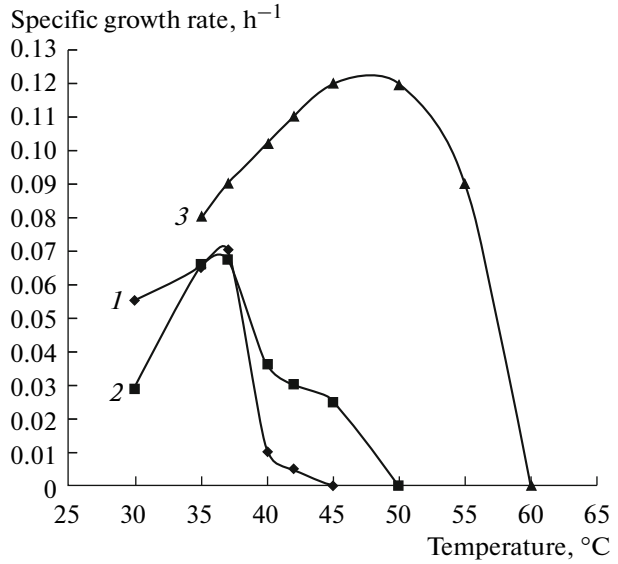

Fig. 3. Specific growth rates of the strains *At. ferrooxidans* OL10-01 (*1*), *L. ferriphilum* OL10-02 (*2*), and *S. thermosulfidooxidans* OL10-03 (*3*) in 9K medium at different temperatures.

40°C, pH decreased from 2.2 to 2.0, indicating low rates of oxidation. At pH 2.0, active formation of endospores began. No sulfur oxidation occurred after the second transfer, although growth continued due to YE consumption. Iron was not oxidized under autotrophic and mixotrophic conditions. Members of the genus *Alicyclobacillus* are known to utilize both organic and inorganic (ferrous iron, sulfur, and various sulfide minerals) substrates for mixotrophic growth [26].

The bacterium *Ac. cryptum* OL10-06 was isolated on the medium with elemental sulfur and YE, but no pH decrease was observed during growth, i.e., the inorganic substrate was not oxidized (table). The isolate also did not oxidize iron.

Thus, microbiological techniques revealed that members of the genera *Sulfobacillus, Leptospirillum*, and *Ferroplasma* played the main part in the oxidation of inorganic energy substrates (elemental sulfur and ferrous iron). *At. ferrooxidans* OL10-01 (JF891384) actively oxidized sulfur at 35°C. *Alicyclobacillus* sp. OL10-05 (JF891385) and *Acidiphilium* sp. OL10-06 (JF891383) persisted in the reactor pulp due to the presence of organic substrates (the products of ACM metabolism and cell lysis). Consumption of dissolved organic matter (DOM) by heterotrophic and mixotrophic microorganisms has a positive effect on the oxidation of mineral substrates by ACM, since the activity of the latter is inhibited at DOM accumulation in the medium [27].

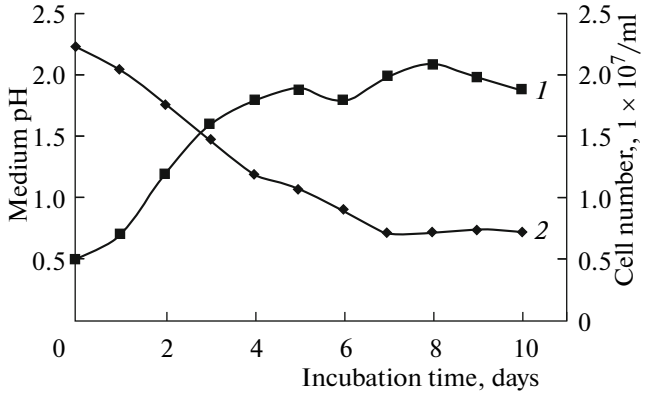

Fig. 4. Oxidation of elemental sulfur by *At. ferrooxidans* OL10-01 at 35°C: cell number, ml^{-1} (*1*) and pH (*2*).

Analysis of the Composition of the Microbial Association Using the 16S rRNA Gene Clone Libraries

In the library of bacterial clones, 51 were obtained and analyzed. All 16S rRNA gene sequences were grouped into two phylotypes belonging to the known genera *Acidithiobacillus* (12 clones) and *Leptospirillum* (39 clones). Ten consensus clone sequences of the genus *Acidithiobacillus* belonged to the phylotype of the strain *At. ferrooxidans* DSM 2392 (Fig. 1). According to the results of the physiological and genotypic investigation of *At. ferrooxidans* strains [28], all the known 16S rRNA gene sequences of this species belong to seven phylogenetically different groups. The sequences obtained in the present work belong to the phylogenetic groups 1–3, which form a single cluster on the phylogenetic tree, while the type strain *At. ferrooxidans* ATCC 23270^T belongs to the phylogenetic group 4. Numerous *At. ferrooxidans* strains with more

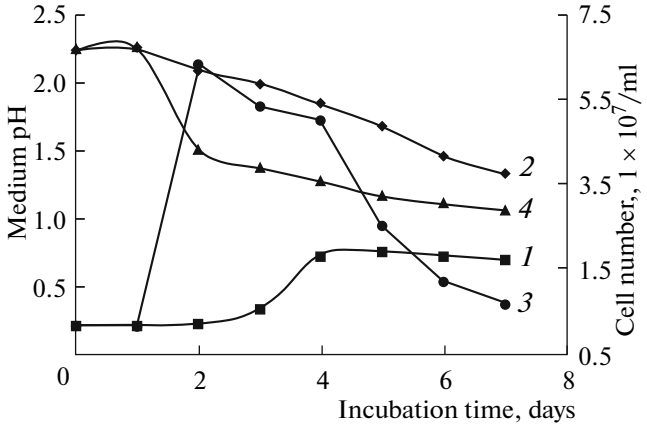

Fig. 5. Oxidation of elemental sulfur by *S. thermosulfidooxidans* OL10-03 at 40°C: cell number, ml^{-1} at 0.002% YE in the medium (*1*), pH at 0.002% YE in the medium (*2*), cell number, ml^{-1} at 0.02% YE in the medium (*3*), and pH at 0.02% YE in the medium (*4*).

or less similar phenotypes were described in the literature. They are all gram-negative rods, acidophilic obligate chemolithoautotrophs, which obtain energy from the oxidation of iron ions and sulfur. The results of 16S genotyping and RAPD analysis demonstrated the heterogeneity of this group of strains [28]. Taxonomic reorganization of the species *At. ferrooxidans* has therefore been proposed more than once, with some strain groups classified as individual species. Considering the above, the cluster including the sequences isolated in the present work and that of *At. ferrooxidans* DSM 2392 possibly comprises bacteria of the new species of this genus, which differs from the type strain *At. ferrooxidans* ATCC 23270^T. The fact that the 16S rRNA gene sequence of the strain *Acidithiobacillus* sp. OL10-01 (JF891384) was clustered together with the type strain *At. ferrooxidans* ATCC 23270^T is an indirect confirmation of this suggestion. Since analysis of the clone library did not reveal such sequences, bacteria physiologically resembling the type strain *At. ferrooxidans* ATCC 23270^T were probably a minor component.

All 39 sequences similar to 16S rRNA genes of *Leptospirillum* formed a single cluster together with the sequence of *L. ferriphilum* BY (EF025341), which was phylogenetically different from the sequences of the type strains *L. ferriphilum* ATCC 49881^T and *L. ferrooxidans* DSM 2705^T (Fig. 2). The sequence of the pure culture *Leptospirillum* sp. OL10-02 (JF891387) belonged to the same cluster. According to the results of phylogenetic analysis, *Leptospirillum* sp. OL10-02 (JF891387) probably belongs to a new species of this genus.

Analysis of the clone library did not reveal *Sulfobacillus* sequences. This may be a result of low *Sulfobacillus* abundance in the microbial community. Different efficiency of DNA recovery from different bacterial species is an alternative explanation. In order to check this hypothesis, a control experiment was carried out with the biomass of the investigated community mixed with the biomass of a pure *Sulfobacillus* culture at the ratios of 10 : 0, 9 : 1, 5 :5, 1 : 9, and 0 :10. Total DNA was then extracted by the method used for the analysis of the community, the 16S rDNA amplificate was obtained, sequenced, and the purity of the sequence obtained was determined. It was discovered that sulfobacilli were detected successfully when they constituted 10% of the total biomass. Therefore, it may be concluded that the number of sulfobacilli in the sample was low, not exceeding 2%. These results don't contradict the data on the rates of iron and sulfur oxidation by the members of three genera: *Sulfobacillus, Acidithiobacillus*, and *Leptospirillum*. At lower cell numbers, sulfobacilli are more active oxidizers of sulfide minerals than acidithiobacilli [29].

The archaeal component of the community was practically a monoculture. Analysis of the 16S rDNA sequence of the strain *Ferroplasma* sp. OL10-04 (JF891386) showed that it was identical to that of the total amplificate, formed a cluster together with the relevant *F. acidiphilum* archaeal sequences, and therefore belonged to the same species.

The results of the present work suggest that a combination of microbiological and molecular ecological techniques for investigation of the biodiversity of natural and anthropogenic communities is advantageous for detection of new physiological and phylogenetic microbial groups.

The cultural techniques revealed a more diverse ACM community (*At. ferrooxidans, L. ferriphilum, S. thermosulfidooxidans, F. acidiphilum, Al. tolerans*, and *Ac. cryptum*) than analysis of the 16S rDNA clone library (*At. ferrooxidans, L. ferriphilum,* and *F. acidiphilum*).

The ACM associations are currently being investigated by cultural and molecular biological techniques. The possibility of determining the composition of a microbial community without isolation of pure microbial cultures is a significant advantage of the PCR-based methods. While obtaining DNA preparations is crucially important for this technique, their quality depends on the abundance of specific microorganisms in the community, their cell wall structure, conditions for efficient lysis, and the specificity of the primers. Moreover, since an investigation of strain polymorphism in ACM demonstrated that phylogenetically-related strains might differ significantly in their physiological characteristics, adequate determination of the functional role of the so-called phylotypes retrieved by molecular ecological studies in the bioleaching processes may be hindered.

The cultural techniques make it possible to obtain pure cultures of the known microorganisms in selective media even at low concentrations in the original community. These techniques, however, are not suitable for uncultured microorganisms. The present work shows that new microorganisms identified by molecular biological techniques are not always isolated on standard media.

Thus, the combination of cultural and molecular biological techniques results in the most complete picture of the species diversity of the ACM associations, and of the role of each microorganism in the community. Investigation of the physiological characteristics of microorganisms suggests conclusions concerning the interspecific interactions in the ACM associations, which are important for the understanding of their functioning in natural and technogenic environments.

ACKNOWLEDGMENTS

This work was supported by the state contract of the Ministry of Science and Education of the Russian Federation no. 02.552.11.7073 and the Russian Foundation for Basic Research, grant no. 10-04-00589.

REFERENCES

1. van Aswegen, P.C., van Niekerk, J., and Olivier, W., The BIOX[TM] Process for the Treatment of Refractory Gold Concentrate, in *Biomining*, Rawlings, D. E. and Johnson, B.D., Eds., Berlin: Springer, 2007, pp. 1–35.
2. Karavaiko, G.I., Dubinina, G.A., and Kondrat'eva, T.F., Lithotrophic Microorganisms of the Oxidative Cycles of Sulfur and Iron, *Mikrobiologiya,* 2006, vol. 75, no. 5, pp. 593–629 [*Microbiology* (Engl. Transl.), vol. 75, no. 5, pp. 512–545].
3. Norris, P.R., Acidophile Diversity in Mineral Sulfide Oxidation, in *Biomining*, Rawlings, D. E. and Johnson, B.D., Eds., Berlin: Springer, 2007, pp. 199–216.
4. Goebel, B.M. and Stackebrandt, E., Cultural and Phylogenetical Analysis of Mixed Microbial Populations Found in Natural and Commercial Bioleaching Environments, *Appl. Environ. Microbiol.*, 1994, vol. 60, no. 5, pp. 1614–1621.
5. Dew, D.W., Lawson, E.N., and Broadhurst, J.L., The BIOX[R] Process for Biooxidation of Gold-Bearing Ores or Concentrates, in *Biomining: Theory, Microbes and Industrial Processes*, Rawlings, D.E., Ed., Georgetown, TX: Springer /Landes Bioscience, 1997, pp. 45–80.
6. Rawlings, D.E., Coram, N.J., Gardner, M.N., and Deane, S.M., *Thiobacillus caldus* and *Leptospirillum ferrooxidans* Are Widely Distributed in Continuous Flow Biooxidation Tanks Used to Treat a Variety of Metal Containing Ores and Concentrates, in *Biohydrometallurgy and the Environment: Toward the Mining of the 21st Century. Part A.*, Amils, R. and Ballester, A., Eds., Amsterdam: Elsevier, 1999, pp. 777–786.
7. Coram, N.J. and Rawlings, D.E., Molecular Relationship between Two Groups of the Genus *Leptospirillum* and the Finding That *Leptospirillum ferriphilum* sp. nov. Dominates South African Commercial Biooxidation Tanks That Operate at 40°C, *Appl. Environ. Microbiol.*, 2002, vol. 68, no. 2, pp. 838–845.
8. Dopson, M. and Lindstrom, E.B., Analysis of Community Composition During Moderately Thermophilic Bioleaching of Pyrite, Arsenical Pyrite, and Chalcopyrite, *Microbial Ecol.,* 2004, vol. 48, pp. 19–28.
9. d'Hugues, P., Battaglia-Brunet, F., Clarens, M., and Morin, D., Microbial Diversity of Various Metal-Sulphides Bioleaching Cultures Grown Under Different Operating Conditions Using 16S rDNA Analysis, *Proc. 15th Int. Biohydrometallurgy Symp. (IBS 2003), Part II*, Tsezos, M., Remoudaki, E., and Hatzikioseyian, A., Eds., Natl. Tech. Univ. Athens, 2003, pp. 1313–1324.
10. Sovmen, V.K., Gus'kov, V.N., Belyi, A.V., Kuzina, Z.P., Drozdov, S.V., Savushkina, S.I., Maiorov, A.M., and Zakrevskii, M.P., *Pererabotka zolotonosnykh rud s primeneniem bakterial'nogo okisleniya v usloviyakh Krainego Severa* (Processing of Gold-Bearing Ores by Bacterial Bioleaching under Arctic Conditions), Novosibirsk: Nauka, 2007.
11. Kondrat'eva, T.F., Melamud, V.S., Aslanukov, R.Y., Voronina, O.B., and Karavaiko, G.I., Chemolitotrophic Bacterial Community in the Pulp of a Pilot Plant during Gold-Arsenic Pyrrhotite Concentrate Processing, in *Biotechnology and the Environment Including Biogeotechnology*, Zaikov, G.E., Ed., New York: Nova Science, 2004, pp. 147–159.
12. Sovmen, V.K., Karavaiko, G.I., Kondrat'eva, T.F., Pivovarova, T.A., Belyi, A.V., Lipatova, T.V., and Gish, A.A., RF Patent no. 2332455 (2006).
13. Ageeva, S.N., Kondrat'eva, T.F., and Karavaiko, G.I., Phenotypic Characteristics of *Thiobacillus ferrooxidans* Strains, *Mikrobiologiya,* 2001, vol. 70, no. 2, pp. 186–194 [*Microbiology* (Engl. Transl.), vol. 70, no. 2, pp. 186–194].
14. Tsaplina, I.A., Bogdanova, T.I., Kondrat'eva, T.F., Melamud, V.S., Lysenko, A.M., and Karavaiko, G.I., Genotypic and Phenotypic Polymorphism of Environmental Strains of the Moderately Thermophilic Bacterium *Sulfobacillus sibiricus, Mikrobiologiya*, 2008, vol. 77, no. 2, pp. 178–187 [*Microbiology* (Engl. Transl.), vol. 77, no. 2, pp. 151–158].
15. Silverman, M.P. and Lungren, D.C., Study on the Chemoautotrophic Iron Bacterium *Ferrobacillus ferrooxidans.* An Improved Medium and Harvesting Procedure for Securing High Cell Yield, *J. Bacteriol.*, 1959, vol. 77, no. 5, pp. 642–647.
16. Melamud, V.S. and Pivovarova, T.A., Specific Features of the Growth of the Type Strain of *Sulfobacillus thermosulfidooxidans* in Medium 9K, *Prikl. Biokhim. Mikrobiol.*, 1998, vol. 34, no. 3, pp. 309–315 [*Appl. Biochem. Microbiol.* (Engl. Transl.), vol. 34, no. 3, pp. 281–286].
17. Golyshina, O.V., Pivovarova, T.A., Karavaiko, G.I., Kondrat'eva, T.F., Moore, E.R.B., Abraham, W., Lünsdorf, H., Timmis, K.N., Yakimov, M.M., and Golyshin, P.N., *Ferroplasma acidiphilum* gen. nov., sp. nov., an Acidophilic, Autotrophic, Ferrous-Iron-Oxidizing, Cell-Wall-Lacking, Mesophilic Member of the *Ferroplasmaceae* fam. nov., Comprising a Distinct Lineage of the *Archaea, Int. J. Syst. Evol. Microbiol.*, 2000, vol. 50, pp. 997–1006.
18. Reznikov, A.A., Mulikovskaya, E.P. and Sokolov, I.Yu., *Metody analiza prirodnykh vod* (Analytical Methods for Natural Waters), Moscow: Nedra, 1970.
19. Bulygina, E.S., Kuznetsov, B.B., Marusina, A.I., 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].
20. Lane, D.J., 16S/23S Sequencing, in *Nucleic Acid Techniques in Bacterial Systematics*, Stackebrandt, E. and Goodfellow, M., Eds., Chichester: Wiley, 1991, pp. 115–175.
21. Kolganova, T.V., Kuznetsov, B.B., and Tourova, T.P., Designing and Testing Oligonucleotide Primers for Amplification and Sequencing of Archaeal 16S rRNA Genes, *Mikrobiologiya,* 2002, vol. 71, no. 2, pp. 283–286 [*Microbiology* (Engl. Transl.), vol. 71, no. 2, pp. 243–246].
22. Sanger, F., Nicklen, S., and Coulson, A.R., DNA Sequencing with Chain-Terminating Inhibitors, *Proc. Natl. Acad. Sci. USA*, 1977, vol. 84, pp. 5463–5467.
23. Thompson, J.D., Higgins, D.G., and Gibson, T.J., CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence

Weighting, Positions-Specific Gap Penalties and Weight Matrix Choice, *Nucleic Acids Res.*, 1994, vol. 22, no. 22, pp. 4673–4680.

24. Van de Peer, Y. and De Wachter, R., TREECON for Windows: a Software Package for the Construction and Drawing of Evolutionary Trees for the Microsoft Windows Environment, *Comput. Applic. Biosci.*, 1994, vol. 10, pp. 569–570.
25. Tsaplina, I.A., Zhuravleva, A.E., Ismailov, A.D., Zakharchuk, L.M., Krasil'nikova, E.N., Bogdanova, T.I., and Karavaiko, G.I., The Dependence of Intracellular ATP Level on the Nutrition Mode of the Acidophilic Bacteria *Sulfobacillus thermotolerans* and *Alicyclobacillus tolerans, Mikrobiologiya*, 2007, vol. 76, no. 6, pp. 742–751 [*Microbiology* (Engl. Transl.), vol. 76, no. 6, pp. 654–663].
26. Karavaiko, G.I., Bogdanova, T.I., Tourova, T.P., Kondrat'eva, T.F., Tsaplina, I.A., Egorova, M.A., Krasil'nikova, E.N., and Zakharchuk, L.M., Reclassification of *"Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans"* Strain K1 as *Alicyclobacillus tolerans* sp. nov. and *Sulfobacillus disulfidooxidans* (Dufresne et al., 1996) as *Alicyclobacillus disulfidooxidans* comb. nov., and Emended Description of the Genus *Alicyclobacillus, Int. J. Syst. Evol. Microbiol.*, 2005, vol. 55, pp. 941–947.
27. Okibe, N. and Johnson, D.B., Biooxidation of Pyrite by Defined Mixed Cultures of Moderately Thermophilic Acidophiles in pH-Controlled Bioreactors: Significance of Microbial Interactions, *Biotechnol. Bioeng.,* 2004, vol. 87, no. 5, pp. 574–583.
28. Yong-Qing, Ni., Kai-Yu, He., Jing-Ting, Bao., Yuan, Yang., Dong-Shi, Wan., and Hong-Yu, Li., Genomic and Phenotypic Heterogeneity of *Acidithiobacillus* spp. Strains Isolated from Diverse Habitats in China, *FEMS Microbiol. Ecol.*, 2008, vol. 64, pp. 248–259.
29. Melamud, V.S., Prospects for the Application of Moderately Thermophilic Sulfide-Oxidizing Bacteria in Gold Biohydrometallurgy, *Tsvetn. Metall.*, 2000, no. 8, pp. 30–33.