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EXPERIMENTAL ARTICLES

Changes in the Species Composition of a Thermotolerant Community of Acidophilic Chemolithotrophic Microorganisms upon Switching to the Oxidation of a New Energy Substrate

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] *Bioengineering Center, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 1, Moscow, 117312 Russia*

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Abstract—Construction and analysis of the 16S rDNA clone libraries was used to investigate the species composition of two thermotolerant communities of acidophilic chemolithotrophic microorganisms (ACM) isolated from the pulp of laboratory reactors used for oxidation of different gold-containing ore concentrates. The first community was formed during oxidation of the pyrite–arsenopyrite ore concentrate from the Kyuchus deposit. The clones of the bacterial component of this community belonged to the genera *Sulfobacillus* (32 clones) and *Leptospirillum* (33 clones). The *Sulfobacillus* clones belonged to three groups: *Sb. thermosulfidooxidans, Sb. benefaciens, and Sb. thermotolerans*. All *Leptospirillum* clones were closely related to *L. ferriphilum*. All clones of the archaeal component belonged to *Ferroplasma acidiphilum*. The microorganisms of this community were used as inoculum for biooxidation of a different mineral concentrate, the pyrrhotite-containing pyrite–arsenopyrite ore concentrate from the Olympiadinskoe deposit, and the structure of the community formed in the process was investigated. The clones of the bacterial component of the second community also belonged to the genera *Sulfobacillus* (14 clones) and *Leptospirillum* (48 clones). The *Sulfobacillus* clones belonged to the species *Sb. thermosulfidooxidans* (13 clones) and *Sb. thermotolerans* (1 clone). All *Leptospirillum* clones were closely related to *L. ferriphilum*. All clones of the archaeal component belonged to *Ferroplasma acidiphilum*. During the adaptation of the community to a new oxidized mineral substrate, both the composition and the ratio of the constituent microbial species changed.

Keywords: tank bioleaching, sulfide ore concentrate, acidophilic chemolithotrophic microbial community, *Leptospirillum, Sulfobacillus, Ferroplasma*, 16S rDNA clone libraries

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Since the rates of the oxidation reactions increase at elevated temperatures, moderately thermophilic and thermotolerant acidophilic chemolithotrophic microorganisms (ACM) are promising objects for biohydrometallurgical recovery of nonferrous and noble metals from sulfide ores. Presently, industrial biooxidation processes are usually carried out at the temperatures optimal for the moderately thermophilic and thermotolerant microorganisms [1]. Thus, microbial communities developing in the course of biooxidation of sulfide minerals at 40–55°C attract special attention. The strains of *Leptospirillum ferrooxidans, L. ferriphilum*, and *Acidithiobacillus caldus* usually predominate in the processes of tank biooxidation at 40–45°C, while *At. ferrooxidans, At. thiooxidans*, and *Sulfobacillus* spp. are also present. At 45–55°C, *At. caldus, Sulfobacillus* spp., and *Acidimicrobium* spp. predominate. Moderately thermophilic and thermotolerant ACM are briefly characterized in Table 1, while the composition of the ACM communities from the reactor pulp during bioleaching of ore concentrates is listed in Table 2. These data show that the species composition of thermoacidophilic microbial communities depends critically upon the temperature and the mineralogical characteristics of the energy substrate. Changes in the dominant species within the communities during biooxidation and replacement of inoculated microorganisms by the autochthonous ones were reported in a number of publications [8, 9].

This research was concentrated on the microbial community oxidizing sulfide minerals during gold recovery from the pyrite–arsenopyrite concentrate of gold–arsenic ore of the Kyuchus deposit [10]. The process was carried out under a variable temperature regime. The scheme of continuous bacterial–chemical oxidation of refractory gold-containing pyrite–arsenopyrite concentrates under a variable temperature mode was originally suggested by Melamud [11]. According to this scheme, the first stage is carried out

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

Table 1. Characteristics of moderately thermophilic and thermotolerant acidophilic chemolithotrophic microorganisms

Parameter	Microorganisms				
	Sulfobacillus	*Leptospirillum*	*Acidimicrobium*	*Ferroplasma*	*At. caldus*
Upper temperature growth limit, °C	60	50	55	60	52
Oxidized substrates	Fe^{2+}, sulfur and sulfur compounds, sulfide minerals	Fe^{2+}, sulfide minerals	Fe^{2+}, sulfide minerals	Fe^{2+}, sulfide minerals	Sulfur and sulfur compound
Organic substrate requirement	Usually require organic compounds, but this requirement varies for different strains	Autotroph	Use organic compounds, but are capable of autotrophic growth	Usually require organic compounds	Autotroph

Table 2. Species composition of the community of acidophilic chemolithotrophic microorganisms in reactors for tank bioleaching/biooxidation of sulfide ore concentrates

Concentrate composition	Temperature, °C	Community composition	Reference
Pyrite, arsenopyrite	40	*L. ferriphilum, At. caldus*, *At. ferrooxidans*	[2]
Pyrrhotite, pyrite, arsenopyrite	40	*Acidiferrobacter thiooxidans, L. ferriphilum, Sb. thermosulfidooxidans*, *F. acidiphilum, At. ferroxidans, Alicyclobacillus tolerans, Acidiphilium cryptum*	[3]
Cobalt-containing pyrite	40	*L. ferriphilum, At. caldus, F. acidiphilum, Sb. benefaciens*	[4]
Pyrite, chalcopyrite, sphalerite	45	*At. caldus, L. ferriphilum, Sulfobacillus* sp.	[5]
Pyrite, arsenopyrite, chalcopyrite	45	*At. caldus, Sb. thermosulfidooxidans*, *"Sb. montserratensis"*	[6]
Pyrite, pentlandite, violarite, pyrrhotite	49	*At. caldus, Acidimicrobium* sp., *Sulfobacillus* sp.	[7]
Pyrrhotite, pyrite, arsenopyrite	50	*Sb. thermosulfidooxidans* HT-1, *Sb. thermosulfidooxidans* HT-3	[8]

Table 3. Characteristics of the mineral substrates

Substrate, deposit	Minerals of the substrate	Geographic location of the deposit
Kyuchus deposit ore concentrate	Pyrite, arsenopyrite, antimonite	Northern Sakha Republic
Olympiadinskoe deposit ore concentrate	Pyrrhotite, pyrite, arsenopyrite, antimonite	Northern Krasnoyarsk krai

at 30°C by mesophilic bacteria *At. ferrooxidans,* while the second stage is carried out by *Sb. thermosulfidooxidans* at 42°C. The rate of oxidation of sulfide minerals increased severalfold, since mixotrophic moderately thermophilic sulfobacilli with the rates of oxidation of sulfide minerals higher than those of acidithiobacilli used the exometabolites and lysis products of the mesophilic autotrophs as carbon sources [12].

The goal of the present work was to investigate the effect of the change of the characteristics of the oxidized mineral substrate on the species composition of the microbial community.

MATERIALS AND METHODS

The subject of research was the community of moderately thermophilic and thermotolerant microorganisms from the reactor pulp of a laboratory reactor for two-stage biooxidation of the Kyuchus deposit pyrite–arsenopyrite gold–arsenic ore concentrate. The concentrate contained the following: Au, 18 g/t; Ag, 2.14 g/t; Fe, 5.53%; As, 3.03%; Sb, 4.1%; S, 5.58%; and Hg, 0.25%. The mineralogical composition of the concentrate is presented in Table 3. The process was carried out under a variable temperature regime (34–36 and 38–42°C for the first and second stage, respectively) at pH 1.65–1.75. The solid phase content in the pulp was 16% [10]. An enrichment culture was obtained from the pulp of the last reactor at the end of the process. For this purpose, 100 mL of the 9KS medium [13] containing 1 g of the Kyuchus deposit ore concentrate was inoculated with 10 mL of the pulp. The culture was grown on a shaker at 40°C. The structure of this enrichment was investigated using molecular genetic techniques.

The biomass of this enrichment culture was used as inoculum for biooxidation of the pyrite–arsenopyrite gold–arsenic ore concentrate from the Olympiadinskoe deposit (Au, 105 g/t; Ag, 0.98 g/t; Fe, 19.2%; As, 7.01%; Sb, 5.30%; S, 17.2%; Ca, 5.25%; and Mg, 0.70%). The mineralogical composition of the concentrate is shown in Table 3. The process was carried out in a 1-L laboratory reactor in a semicontinuous mode at pulp density 10 g of the concentrate per 1 L of the 9K medium mineral base. The flow rate was 100 mL/day. The temperature was maintained at 39–40°C, pH 1.60–1.65.

DNA isolation from bacterial biomass was carried out according to [14]. The concentration of the DNA preparations obtained by this technique was 30–50 μg/mL. RNA was present in trace amounts, below 1% (results of electrophoretic analysis are not presented).

Amplification of 16S rRNA genes and cloning and sequencing of the PCR products. Universal primers [15] were used for polymerase chain reaction, cloning of the 16S rRNA gene fragments, and sequencing of the clonal inserts of eubacterial origin. The original primer system [16] was used for polymerase chain reaction and sequencing of the archaeal component of the community. 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 each relevant primer, and 3 U BioTaq DNA polymerase (Dialat, Russia).

The PCR was carried out on a Gradient MasterCycler DNA amplifier (Eppendorf, Germany) according to the following protocol: first cycle, 94°C × 9 min, 55°C × 1 min, 72°C × 2 min; 30 cycles of 94°C × 1 min, 55°C × 1 min, 72°C × 2 min; and final annealing for 7 min at 72°C.

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

Isolation of PCR products from low-melt agarose and their purification were carried out 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 [17] on an ABI PRIZM 3730 automatic sequencer (Applied Biosystems, United States) using the Big Dye Terminator v. 3.1 reagent kit (Applied Biosystems, United States) according to the manufacturer's recommendations. Both internal and external primers were used for sequencing; reading was carried out in two directions.

Analysis of the 16S rRNA gene sequences. Primary analysis of the similarity between the nucleotide sequences of the 16S rRNA genes was carried out using the BLAST server (http://www.ncbi.nlm.nih.gov/blast). The sequences were aligned with the relevant sequences of related bacteria using the CLUSTAL W software package [18]. Phylogenetic trees were constructed using the methods implemented in the TREECONW software package [19]. Statistical reliability of the branching order was determined by the bootstrap method analyzing 1000 alternative trees.

RESULTS AND DISCUSSION

Analysis of the structure of the microbial community biooxidizing the Kyuchus ore concentrate. The fragments of 16S rRNA genes of 65 independent clones analyzed for the bacterial component fell into two groups. The sequences of 32 clones belonged to various *Sulfobacillus* species forming three clusters: *Sb. thermosulfidooxidans* (18 clones), *Sb. benefaciens* (2 clones), and *Sb. thermotolerans* (12 clones) (Fig. 1). The sequences of 33 clones were related to *Leptospirillum* sequences and formed a homogeneous group close to the species *L. ferriphilum* (Fig. 2). For the archaeal

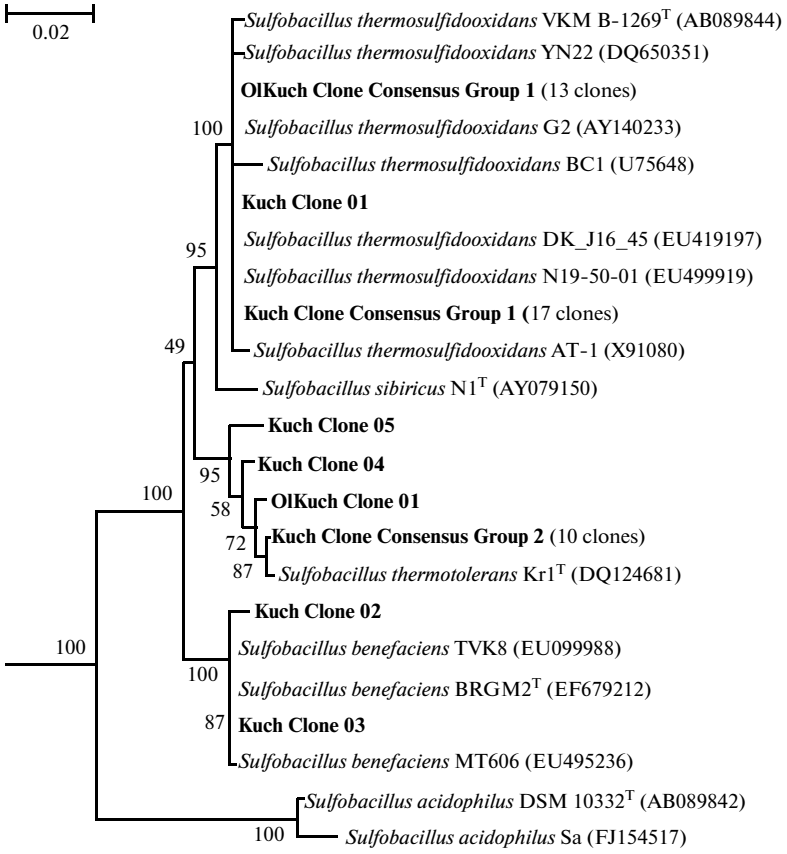

Fig. 1. Summary tree of the 16S rRNA gene sequences of *Sulfobacillus* species demonstrating the phylogenetic position of the consensus phylotypes and isolated strains. The dendrogram was constructed using the neighbor-joining algorithm by comparison of the 781-bp fragments and analysis of 1000 alternative trees. The significance of the branching order is indicated in percent. The evolutionary distance scale is shown at the top. The sequences determined in the present work are in boldface. The sequences related to the community responsible for biooxidation of the Kyuchus ore concentrate are designated as **Kuch**, while those responsible for the oxidation of the Olympiadinskoe ore concentrate are designated as **OlKuch**. The 16S rRNA gene sequence of the type strain *Alicyclobacillus disulfidooxidans* DSM 12064 (GenBank no. AB089843) was used as an outgroup.

component, 37 independent clones were analyzed. All of them corresponded to *F. acidiphilum*.

Analysis of the structure of the microbial community biooxidizing the Olympiadinskoe deposit ore concentrate. For the bacterial component of the community, 16S rRNA fragments of 62 clones were analyzed. According to the results of phylogenetic analysis, they formed two groups: 14 clones of the *Sulfobacillus* group and 48 clones of the *Leptospirillum* group. In the *Sulfobacillus* clone group, two phylotypes were found, one (13 clones) forming a cluster with *Sb. thermosulfidooxidans* sequences, including that of the type strain, and another (1 clone) clustering together with the *Sb. thermotolerans* type strain (Fig. 1). Phylogenetic analysis of the *Leptospirillum* group revealed one phylotype most closely related to *L. ferriphilum* (Fig. 2). All archaeal sequences belonged to *F. acidiphilum*.

Thus, during the oxidation of a substrate with different mineralogical characteristics, the species composition of the microbial community changed (the species *Sb. benefaciens* was absent), as well as the qualitative ratio of the species. For example, the share of *Leptospirillum* clones in the community oxidizing the Kyuchus ore concentrate was about 50% of the total number of bacterial clones, while during biooxidation of the Olympiadinskoe ore concentrate it increased to 77%. Under the same conditions, the share of *Sulfobacillus* clones decreased from 50 to 23%. *Sb. benefaciens* clones disappeared, while the *Sb. thermotolerans* clones, which constituted a significant fraction of the total number of *Sulfobacillus* clones (12 out of 32), became a minor component (1 out of 14 *Sulfobacillus* clones).

The results of the present work may be used to explain the fact that prolonged continuous biooxidation of sulfide ores results in a gradual change in the composition of microbial communities. Importantly, these processes are not carried out aseptically, so autochthonous strains continuously arriving into the reactor pulp may gradually displace the strains used as

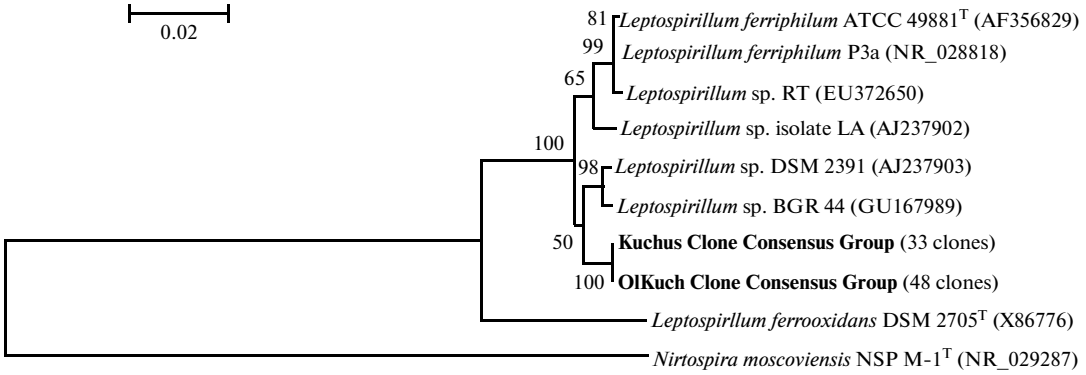

Fig. 2. Summary tree of the 16S rRNA gene sequences of *Leptospirillum* species demonstrating the phylogenetic position of the consensus phylotypes and isolated strains. The dendrogram was constructed using the neighbor-joining algorithm by comparison of the 785-bp fragments and analysis of 1000 alternative trees. The significance of the branching order is indicated in percent. The evolutionary distance scale is shown at the top. The sequences determined in the present work are in boldface. The sequences related to the community responsible for biooxidation of the Kyuchus ore concentrate are designated as **Kuch**, while those responsible for the oxidation of the Olympiadinskoe ore concentrate are designated as **OlKuch**. The 16S rRNA gene sequence of the type strain *Nitrospira moscoviensis* NSP M-1^T (GenBank no. NR_029287) was used as an outgroup.

inocula [8, 9]. It is also known that the same deposit may contain the zones with ores differing in their mineralogical and chemical composition. Thus, oxidation of the nonstandard concentrates from the currently produced ore may result in adaptive changes within a microbial community.

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