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EXPERIMENTAL ARTICLES

Bacteria of the Sulfur Cycle in the Sediments of Gold Mine Tailings, Kuznetsk Basin, Russia

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Abstract—The number and diversity of culturable microorganisms involved in sulfur oxidation and sulfate reduction were investigated in the oxidized sediments of gold mine tailings, Kuznetsk Basin, Russia. The sediments had a low pH (2.4–2.8), high SO_4^{2-} content (up to 22 g/l), and high concentrations of dissolved metals. The arsenic content was as high as 1.9 g/l. Bacterial phylogeny in microcosms was investigated by amplification of 16S rRNA gene fragments with subsequent denaturing gradient gel electrophoresis (DGGE). Spore-forming bacteria *Desulfosporosinus* were the only bacteria revealed for which the capacity for dissimilatory sulfate reduction is known. Strain *Desulfosporosinus* sp. DB was obtained in pure culture, and it was phylogenetically remote from other cultured and uncultured members of the genus. No sulfate-reducing members of the *Deltaproteobacteria* were detected. The *Firmicutes* members were the most numerous phylotypes in the microcosms, including a separate cluster with the similarity to *Pelotomaculum* not exceeding 94%. *Acidithiobacillus ferrooxidans* and *A. caldus* were found in anaerobic and microaerophilic microcosms. The number of sulfate reducers did not exceed 9.5×10^2 cells/ml.

Key words: denaturing gradient gel electrophoresis, acid mine drainage, sulfate-reducing bacteria, gold mine tailings, *Acidithiobacillus, Desulfosporosinus, Pelotomaculum, Thermincola.*

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Mining and processing of the sulfide metal ores results in high amounts of solid waste. In gold processing, the volume of the concentrate obtained after the concentration and flotation stages often does not exceed 1% of the total amount of processed ore [1]. The waste, including its fine-grained fractions, is not processed and is disposed close to the production site. The so-called "tailings" comprise the waste with high concentrations of associated sulfides (after the concentration stage) and the flotation and cyanidation waste. Large-scale oxidation of residual sulfides in the tailings of the gold and sulfide ore is a source of acidic drainage water with high concentrations of metal ions and is therefore a significant environmental hazard. The chemolithotrophic bacteria that utilize sulfur and iron as electron donors contribute to the oxidation and solubilization of metals; on the other hand, microbial sulfate reduction may be a mechanism for metal precipitation in such ecosystems [2].

The goal of the present work was to investigate the number and diversity of culturable microorganisms involved in sulfur oxidation and sulfate reduction in the oxidized sediments of the tailings of the Novyi Berikul' gold mine where our preliminary data indicated significant bacterial sulfate reduction (Karnachuk et. al., unpublished data).

MATERIALS AND METHODS

The sediments of the tailings of the former Novyi Berikul' gold mine, Kuznetsk Basin, were the object of investigation; gold mining was carried out there in 1933–1941 and 1949–1951 [3]. Milling, flotation, and cyanidation of the ore were carried out at the shore of the Mokryi Berikul' river. The wastes were collected at the same site, in tailings separated by a tailings dam. Some of the tailings sediments were removed in late

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1990s and covered with barren rock. The remaining sediments, however, still undergo oxidation, resulting in numerous seeps with low pH and high concentrations of dissolved metals.

The upper (oxidized) sediment layer and near-bottom water were sampled on July 11, 2006. The temperature, pH, and water conductivity were determined on the site with a HANNA HI 8314F ionometer. For other chemical analyses, the water was filtered through 0.45 μm filters and collected into 120-ml polyethylene vials. The concentrations of metal ions were determined in the laboratory of the Norwegian Geological Survey by Inductively coupled plasma mass spectrometry (ICP-MS) and Inductively coupled plasma atomic emission spectroscopy (ICP-AES). Anion concentrations were determined in the same laboratory with a Dionex 120 DX ion exchange chromatograph. Prior to analyses, the samples were stored at 4°C. For determination of metals, the samples were acidified with 0.5% HNO_3 (a. g.) to prevent adsorption/precipitation. Acidified aliquots were analyzed with Thermo Jarrell Ash ICP61 (ICP-AES) and Finnigan Mat with Menhart Nebulized CD-1 (ICP-MS).

The numbers of sulfate-reducing bacteria (SRB) were determined by the most probable number (MPN) method in a freshwater Widdel medium [4] with lactate, acetate, or ethanol as electron donors. Penicillin vials were filled with the medium to capacity; a steel paper clip was used as an additional source of iron and to maintain the low values of redox potential due to formation of cathode hydrogen; it also acted as a nucleation site for iron sulfide. The MPN series were incubated at 28°C. SRB growth was assayed as darkening of the medium due to formation of iron sulfide and as increased sulfide content. All the MPN series were carried out in three replicates. The most probable numbers were calculated using the McCready tables.

Enrichment cultures of SRB were obtained on a Widdel medium from the MPN series. Lactate, acetate, ethanol, and formate were used as growth substrates, and the cultures were incubated at 28°C. For the isolation of pure cultures, the highest dilutions in the MPN series exhibiting SRB growth were used as inocula. Regular transfers to the same medium were carried out, with initial Cu(II) concentration increased to 650 mg/l. Copper was added from a sterile stock solution of $CuSO_4 \cdot 5H_2O$. The purity of the culture was determined by microscopy and by sequencing of a nearly complete 16S rRNA gene sequence.

For microcosm construction, 1 ml of the upper sediment layer and near-bottom water was introduced to a penicillin vial, which was then filled to capacity with the Widdel medium with organic compounds (lactate, acetate, or ethanol). The initial pH in various variants was either 7 or 2; the microcosm was supplemented with 200 mg/l of Cu(II). Some microcosms were incubated under microaerophilic conditions, with the vials filled to half capacity. For microaerophilic microcosms, lactate was the only carbon source; 200 mg/l of Cu(II) was added; the initial pH value was 2. All the vials were sealed with rubber stoppers fixed in place by aluminum hoods. The microcosms were incubated for 3 months at 28°C.

DNA was isolated from the microcosms with an MO BIO PowerSoil DNA Kit (MO BIO Laboratories, Inc., Carlsbad, United States) according to the manufacturer's recommendations. Prior to determination of the phylogenetic position of the cultured microorganisms by denaturing gradient gel electrophoresis (DGGE), a fragment of the 16S rRNA gene corresponding to *E. coli* 341–926 was amplified using the primers GC-BacV3f (5'-GCclump-CCT ACG GGA GGC AGC AG-3') and 907r (5'-CCG TCA ATT CMT TTG AGT TT-3') [5]. The PCR mixture contained the following: 1 μl template DNA (over 50 ng) and 5 μl of 10× DNAzyme DNA Polymerase buffer (Finnzymes) (1.5 μM $MgCl_2$, 1 μl BSA (Fermentas), 0.2 μl 25 μM dNTP mixture (Amersham), 0.25 μl of each primer, and 0.4 μl DNAzyme II DNA Polymerase 2 U/μl (Finnzymes)). Amplification was carried out using a Thermocycler T3000 Biometra®, The Netherlands) according to the following program: initial denaturation, 5 min at 95°C; 30 denaturation cycles, 30 s at 94°C; annealing, 1 min at 50°C; elongation, 2 min at 72°C; and final elongation, 10 min at 72°C. The PCR product was visualized in 1% agarose gel stained with ethidium bromide. DGGE separation of the amplified fragments was carried out using an INGENY phor U-2 system (Ingeny International BV, The Netherlands) in linear gradients of urea and formamide from 20 to 70% (for samples Xa116) and from 30 to 70% (for samples Xa115) in 8% polyacrylamide gel; 100% denaturing conditions were determined as 7 M urea and 40% formamide. Electrophoresis at 100 V was carried out for 22 h at 60°C. After electrophoresis, the gel was stained in the SYBR Gold solution (Invitrogen) according to the manufacturer's instructions and photographed on a 3UV transilluminator with a Kodak camera with the Kodak 1D software package. The bands were excised and incubated in 20 μl of MilliQ water for 12 h at 4°C. The supernatant was then used for PCR reamplification with the same primers without GC-clamps. Amplification conditions were the same, and BSA was not added to the PCR mixture. The sequencing was performed on a commercial basis by Macrogen Ltd, Seoul, Korea.

To determine the nucleotide sequence of the 16S rRNA gene of a pure SRB culture, amplification was carried out with the 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') primers [7]. To obtain template DNA, 1 ml of the culture at the late exponential phase was centrifuged at 9000 rpm for 10 min. The pellet was washed with 1 ml of 1 × PBS buffer. The procedure was repeated three times. After washing, the pellet was resuspended in 100 μl of 1× PBS buffer and frozen at –20°C. The PCR mixture (50 μl) contained 5 μl of 10× PCR buffer with 200 mM $(NH_4)_2SO_4$ (Fermentas), 5 μl

Table 1. Characterization of the sampling site and some physicochemical parameters of the samples

Sample designation	Sampling site and sample description	Water pH	*T*, °C	Conductivity, μS/cm
Xa115	Shallow wetland, not deeper than 10 cm, at the shore of the Mokryi Berikul' river at the site of the former mine tailings, some 50 m from the waste rock repository and former plant for gold ore treatment. The sediment consists of a thin warp layer covering the rock fragments. The warp is of intense orange color. The rock fragments on the wetland shores are also covered with orange sediment from above and have a greenish coloration at the bottom. The warp mixed with near-bottom water was used for the microcosms	2.8	20.9	6700
Xa116	A Small puddle with the water layer over the sediment not exceeding 2 cm at the shore of the Mokryi Berikul' river. The distance from the water edge does not exceed 1 m. The water in the puddle is of intense red color. Orange and white deposits are present on the shores. The upper sediment layer mixed with near-bottom water was used for the microcosms	2.4	20.4	8500

of 2.5 mM $MgCl_2$ (Fermentas), 2.5 μl DMSO, 2.5 μl of dNTP mixture (Fermentas), 0.25 μl of each primer (10 pM), 1 μl of template DNA (over 50 ng), and 0.5 μl *Taq* polymerase (Fermentas). Amplification was carried out in a Mastercycler (Eppendorf) according to the following program: initial denaturation, 20 s at 95°C; six denaturation cycles, 10 s at 95°C; annealing, 20 s at 45°C; elongation, 1.5 min at 72°C; 27 denaturation cycles, 10 s at 95°C; annealing, 20 s at 55°C; elongation, 1.5 min at 72°C; and final elongation, 3 min at 72°C. DNA sequencing was carried out in a Beckman Coulter CEQ 8000 automatic sequencer (State Research Institute for Genetics and Selection of Industrial Microorganisms).

Analysis of the DNA sequences was carried out with the BioEdit software package and BLAST (http://www.ncbi.nlm.nih.gov/) [8]. Phylogenetic analysis was carried out with the ARB software package (http://www.arb-home.de). The sequences were aligned using the FastAlign tool (ARB software package) relative to the sites with a known secondary structure; all alignments were checked manually. The original phylogenetic tree was constructed by the neighbor-joining method for complete or nearly complete 16S rRNA gene sequences. The shorter gene fragments corresponding to phylotypes/bands in the DGGE gel were added using the algorithm of parsimony analysis, which enables introduction of short sequences not affecting the tree structure. Bootstrap analysis based on 1000 replicas was carried out to determine the confidence level for each node. All the DNA sequences were deposited in GenBank NCBI (accession numbers EU737112 and FJ493549-FJ493587).

RESULTS

Physicochemical parameters of the biotopes. Biodiversity of the culturable bacteria of the sulfur cycle was investigated in the sediments of two small wetlands on the territory of the former tailings. Description of the biotopes and characterization of the samples are presented in Table 1. The water had a very low pH (from 2.4 to 2.8). In both samples, high concentrations of sulfate were revealed, 22.8 and 8.45 g/l for Xa116 and Xa115, respectively (Table 2). The content of dissolved iron was also high, 2.0 and 9.1 g/l for Xa115 and Xa116, respectively. In the sample Xa116, the arsenic concentration was extremely high, 1.9 g/l. The concentrations of other dissolved metals (zinc,

Table 2. Concentrations of some anions and cations in wetland water

Concentration of anions/cations/elements, mg/l	Xa115	Xa116
F^-	3.89	5.25
Cl^-	10.2	15.2
Br^-	<5.0	5.23
SO_4^{2-}	8448	22876
Si	75.5	72.4
Al	286	518
Fe	2020	9100
Ti	0.0272	2.23
Mg	267	445
Ca	502	357
Na	25.7	6.89
Mn	32.9	52.5
Cu	12.6	35.2
Zn	133	351
Pb	1.27	8.49
Ni	4.29	4.88
Co	2.35	4.77
Cd	2.03	5.72
Cr	0.347	2.73
As	24.2	1910

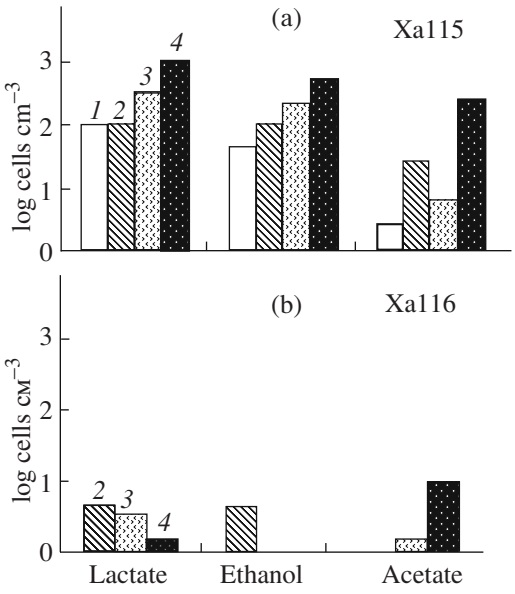

Fig. 1. SRB numbers in Xa115 and Xa116 sediments determined by the MPN method with various electron donors, under different values of pH and copper concentration in the medium: pH 7.2 (*1*); pH 7, 200 mg/l Cu(II) (*2*); pH 2 (*3*); pH 2, 200 mg/l Cu(II) (*4*).

copper, aluminum, cobalt, nickel, cadmium, and lead) were also high. In the water from Xa116, the overall concentration of metal ions was higher than in Xa115.

SRB numbers in the sediments; isolation of enrichment and pure SRB cultures. The maximal number of SRB determined by the MPN method (9.5×10^2 cells/ml) was revealed in the upper sediment layer from Xa115 (Fig. 1a). In the upper sediment layer of Xa116, the number of SRB growing on the experimental substrates was lower and did not exceed 9.5 cells/ml (Fig. 1b). On lactate and ethanol, growth occurred at higher dilutions than on acetate. This tendency was most pronounced for Xa115 samples. Interestingly, in Xa115 samples, the highest SRB numbers were detected under acidic conditions (pH 2) and elevated copper content (at initial Cu(II) concentration of 200 mg/l). The same pattern of higher detected microbial numbers at decreased cultivation pH and increased metal concentration was observed for all organic substrates under study.

The MPN series obtained from the Xa115 sediments were used to establish enrichment cultures. A pure SRB culture was isolated from the enrichment exhibiting good growth on ethanol under elevated copper concentrations. The pure culture was obtained by dilutions in liquid medium with initial Cu(II) concentration increased to 650 mg/l. Under light microscopy, motile rods designated as strain DB were uniform. Sequencing of the 16S rRNA gene confirmed genetic homogeneity of the culture. Phylogenetic analysis of a nearly complete 16S rRNA gene sequence (1473 bp) places the strain within the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*, family *Peptococcaceae*, genus *Desulfosporosinus.* Strain DB is sufficiently remote from the known cultured and uncultured members of this genus. *Desulfosporosinus auripigmenti* (96.6% similarity) is the closest validly described relative (Fig. 2).

Phylogenetic affiliation of the bacteria of the sulfur cycle and other microorganisms from microcosms. After 3 months of cultivation, anaerobic microcosms exhibited pronounced sulfate reduction. The sediment turned black, probably due to precipitation of iron sulfides. In microaerophilic microcosms, an orange ochrous precipitate indicates the development of oxidative processes.

Sequencing and phylogenetic analysis of 16S rRNA gene fragments of the phylotypes separated by DGGE revealed, apart from dissimilatory sulfate reducers, other groups of the domain *Bacteria.* First of all, the phylotypes related to iron-oxidizing *Acidithiobacillus ferrooxidans* should be mentioned (Figs. 2, 3). Aerobic *A. ferrooxidans* were revealed in sulfate-reducing microcosms incubated anaerobically for 3 months. The phylotype DGGE_Xa115_EthpH2Cu200_12 (band 12 on Fig. 4) was revealed in the microcosm from Xa115 sediments with ethanol as an organic substrate, elevated Cu(II) concentration, and acidic incubation medium. Phylotype 12 exhibited 99% similarity to *A. ferrooxidans* CCM4253 (GeneBank accession no. EF465493). The phylotype exhibited 100% similarity to the uncultured clone AY678254 from acidic springs of Yellowstone Park [9]. This cluster comprises other sequences closely related to *A. ferrooxidans* and isolated from the environments contaminated by mine drainage, clone EF446231 from the Rio Tinto filaments (99% similarity) [10] and clone EF464644 from methylmercury-contaminated mine tailings (99% similarity) [Winch et al., unpublished]. The DGGE profiles for other cultivation conditions demonstrated the presence of this organism in all microcosms with low pH (bands 23, 30, 40, 47) (Fig. 4). In Xa116 anaerobic microcosms, another phylotype related to *A. ferrooxidans* was revealed. Phylotype 289 exhibited the highest similarity (98%) to *A. ferrooxidans* strain EF465493 isolated from the Rio Tinto filaments, Spain [10].

In microaerophilic microcosms Xa116, the phylotypes related to another sulfur-oxidizing chemolithotroph, *A. caldus*, were detected (244, 245). In DGGE profiles obtained from anaerobic microcosms, no band corresponded to the intense 245 band from microaerobic microcosms. No phylotypes related to *A. ferrooxidans* were revealed in microaerophilic microcosms.

Phylogenetic analysis of the phylotypes revealed that most of the organisms from Xa115, for which the ability to reduce sulfate is known, belonged to the genus *Desulfosporosinus* (Fig. 2). A number of phylotypes (85, 72, 84, and 42) fell in the same cluster with the recently described species *D. lacus* (98% similarity). *Desulfosporosinus* sp. 44a_T3a isolated from the

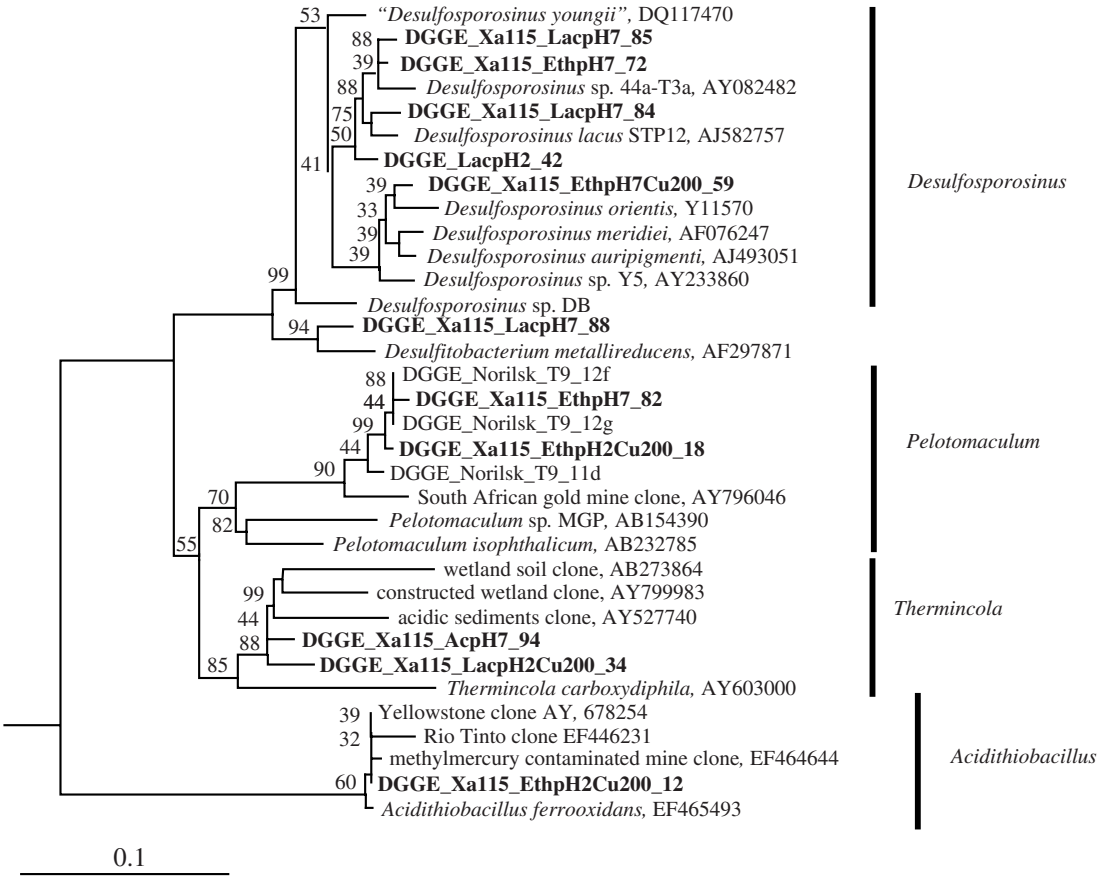

Fig. 2. Phylogenetic position of the DGGE-separated phylotypes from Xa115 microcosms and strain *Desulfosporosinus* sp. DB. The shorter sequences were added using parsimony analysis to the tree constructed using the neighbor-joining method. *Methanobacterium formicicum* AF169245 (not shown on the tree) was used as an outgroup.

biofilms producing ZnS in mine water [11] fell into the same cluster. The similarity between this strain and phylotype 72 was 98%. Two organisms from an Xa116 microcosm (phylotypes 243 and 246) belonged to the same cluster (99% similarity to *D. lacus*) (Fig. 3).

D. orientis was the closest relative (98% similarity) of the organism represented by phylotype 59 (Xa115). In Xa116 microcosms supplemented with ethanol and lactate, a number of phylotypes related to *D. orientis* were also revealed (phylotypes 261, 291, 263, 264, 271, 297, and 260, Fig. 3). These phylotypes were not revealed with acetate as a carbon source or under microaerophilic cultivation conditions.

A number of phylotypes revealed in Xa115 (73, 82, 74, and 18) formed a cluster within *Peptococcaceae* (Fig. 2). Among validly described species, they were most closely related to a syntrophic *Pelotomaculum isophthalicum* (not more than 91% similarity). Most of the organisms detected in Xa116 microcosms belonged to this group (Fig. 3). The similarity between the sequences of Xa116 phylotypes and those of *P. isophthalicum* or *P. shinkii* did not exceed 94%. Most of the phylotypes of this cluster were detected in the microcosms cultivated under microaerophilic conditions.

Some *Firmicutes* were revealed in microcosms Xa115 and Xa116; they belonged to the group with *Thermincola carboxydiphila* as the closest validly described relative. The similarity between the sequences of *T. carboxydiphila* and those of phylotypes 265, 304, and 283 (Xa116) and 34 and 94 (Xa115) did not exceed 93%.

DISCUSSION

The mine tailings studied in the present work are natural environments with extremely low pH. In nature, the ecosystems with such acidic conditions are rare; the known instances are usually associated with active biological oxidation of sulfide ores. Active oxidation of residual sulfides in the tailings of the Berikul' mine is confirmed by extremely high concentrations of sulfate and dissolved iron measured in these environments. A high content of dissolved aluminum results probably from hydrolysis of aluminum silicate minerals under these conditions. High concentrations of metal cations are a characteristic feature of acidic mine waters. However, arsenic concentration determined in the present work (almost 2 g/l) is high even for such environments.

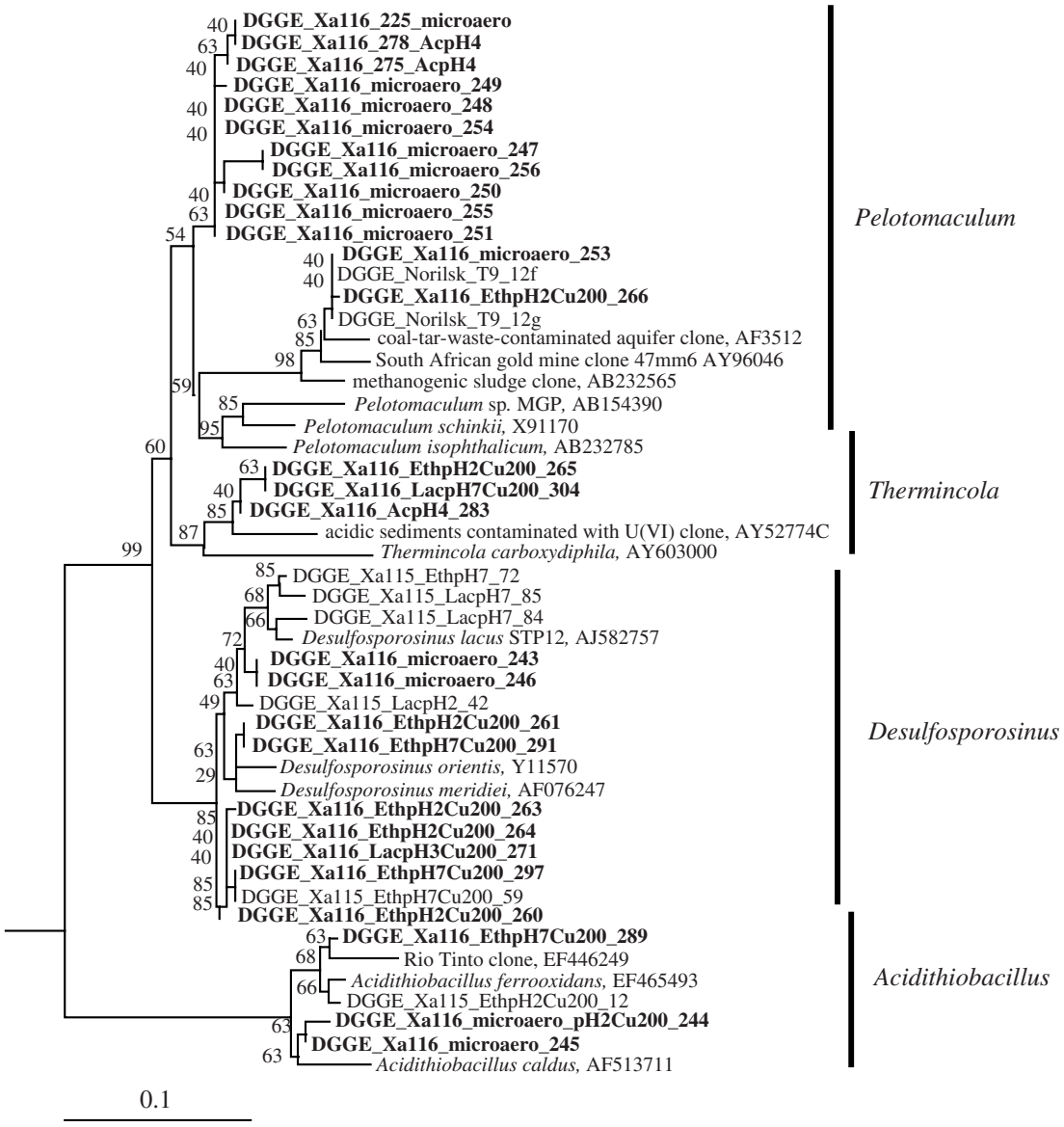

Fig. 3. Phylogenetic position of the DGGE-separated phylotypes from Xa116 microcosms. The shorter sequences were added using parsimony analysis to the tree constructed using the neighbor-joining method. *Methanobacterium formicicum* AF169245 (not shown on the tree) was used as an outgroup.

Arsenopyrite, which is among the major associated sulfide ores of the Berikul' deposit [3], is a probable source of arsenic. The author reported also the presence of sphalerite (ZnS) responsible for the high zinc concentration in the wetlands under study. This ecosystem is similar to the known Rio Tinto habitats where the water is discharged and drains the Pyrite Belt of the Pyrenees [10]. However, while pH in the studied mine tailings was comparable to that of the Rio Tinto, the concentration of the number of metal ions was much higher than the values reported for the Spanish site.

A. caldus revealed in Xa116 sediments may play a specific role in arsenopyrite oxidation and acidification of the medium. Dopson and Lindstrom [12] experimentally confirmed that by oxidation of elemental sulfur and other compounds screening the surface of arsenopyrite, this organism might significantly enhance its dissolution rate. Moreover, since sulfur oxidation by *A. caldis* is a true proton-generating reaction, it may contribute to the decrease in pH.

Five phylogenetically different phyla are presently known to contain the organisms obtaining energy via dissimilatory sulfate reduction [3]. The most numerous groups of such organisms are concentrated in the class *Deltaproteobacteria* and the phylum *Firmicutes.* No members of the *Deltaproteobacteria* were revealed in the sediments of the mine tailings studied in the present work, this is a unique feature of this environment. Both, the phylotypes obtained and the pure SRB culture belonged to spore-forming *Firmicutes.* The experimen-

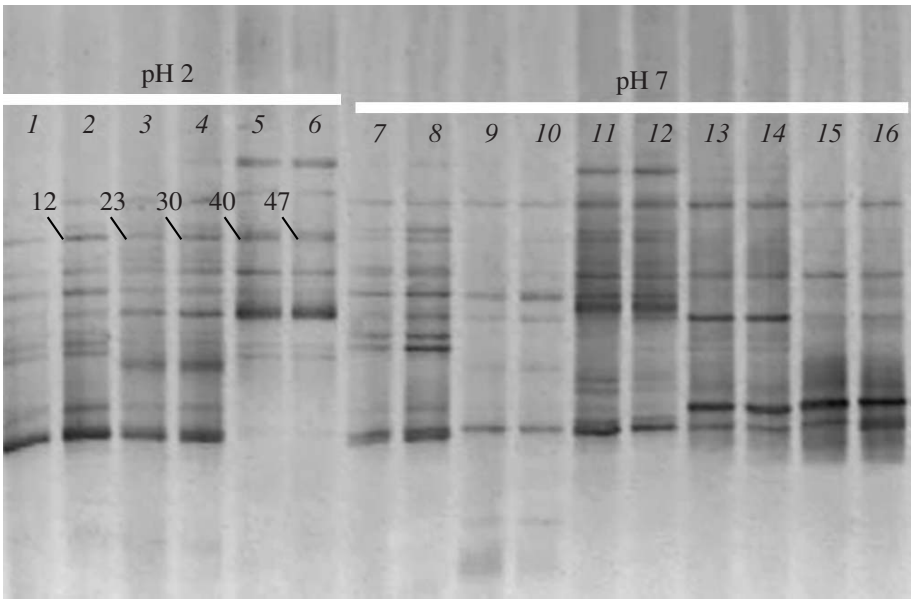

Fig. 4. DGGE profiles of the PCR-amplified 16S rRNA gene fragments from Xa115 microcosms. Lanes: *1, 2*, microcosms with ethanol and 200 mg/l Cu(II); *3, 4*, microcosms with lactate and 200 mg/l Cu(II); *5, 6*, microcosms with lactate without copper; *7, 8*, microcosms with ethanol and 200 mg/l Cu(II); *9, 10*, microcosms with lactate and 200 mg/l Cu(II); *11, 12*, microcosms with ethanol without copper; *13, 14*, microcosms with lactate without copper; *15, 16*, microcosms with acetate without copper. 12, 23, 30, 40, 47—phylotypes nos. on lanes *2–6*, respectively.

tal approach involving microbial cultivation certainly decreased the possibility of detection of all *Bacteria* present in the biotope. However, many *Deltaproteobacteria* are easily cultivated. We have previously detected numerous members of this class in the ecosystems contaminated by metal mining and production, including the sediments of mine tailings [14]. The method of fluorescent in situ hybridization (FISH) used for screening revealed that members of the families *Desulfobulbaceae* and *Desulfovibrionaceae* belonging to the *Deltaproteobacteria* were predominant in enrichment cultures obtained from the sediments of the mine tailings of the Norilsk industrial zone. The domination of sulfate-reducing *Firmicutes* revealed in the present work probably reflects the true ratio of spore-forming and gram-negative SRB in the sediments of this habitat.

Spore-forming microorganisms have evident advantages in the ecosystems with extremely low pH and high concentration of metal ions. Their presence in drainage of an acid mine was reported by other researchers. Labrenz and Banfield [11] revealed *Desulfosporosinus* in microbial biofilms from an abandoned zinc and lead mine only by cultivation techniques. Their library of 16S rRNA gene clones did not contain the sequences of spore-forming SRB. The authors suggest that bacteria were in the sporulation phase and their DNA was therefore less available for isolation. The group of gram-negative *Desulfobacteriaceae* was the most numerous in the investigated biofilms. Strain *Desulfosporosinus* sp. 44a-T3a isolated by Labrenz and Banfield belongs to the cluster comprising some of the phylotypes obtained in the present work (Figs. 2, 3). A number of uncultured members of *Desulfosporosinus* closely related to our phylotypes were detected in metal-contaminated environments. For example, clone A1_bac, falling into the same cluster with strain DB, was obtained from the sediments of methylmercury-contaminated mine tailings [Winch et al., unpublished data]. Other instances are known of *Desulfosporosinus* detection in metal-contaminated environments by both cultivation [15] and molecular techniques [16–18]. *Desulfosporosinus* may use arsenic as an electron acceptor [19]. Sulfate-reducing *Desulfosporosinus* probably plays a specific role in the environments with low pH and high concentrations of metal ions.

The physiological role of a group of gram-negative organisms most closely related to *Thermincola* is still unclear. Capacity for dissimilatory sulfate reduction was not described for these bacteria. *T. carboxydiphila* is a thermophilic alkaliphile [20], and emergence of this phenotype in an extremely acidic Siberian biotope is highly improbable. Since the similarity between our phylotypes and *T. carboxydiphila* was relatively low, not exceeding 93%, significant phenotypic differences may be expected. These bacteria are probably capable of iron reduction. All the presently known *Thermincola* species different from the type strain can reduce ferric iron [21].

Although the redox potential was not determined during sampling, high content of ochre (demonstrated by the bright orange coloration of the sediments) unequivocally confirms that the upper sediment layers are oxidized. A high content of sulfate and iron suggests extensive development of oxidative processes.

Sulfate reduction is traditionally considered a strictly anaerobic process. Although modern data on the physiology and biochemistry of sulfate-reducing bacteria indicate their high tolerance to oxygen, ecological and biogeochemical research on these bacteria is still focused on anaerobic environments. It is traditionally believed that in the ecosystems associated with mining drainage, SRB may be involved in the biogeochemical processes in the anaerobic zone, below the oxidized surface layers. We have previously reported noticeable rates of sulfate reduction in the wetland sediments with positive *Eh* values in the Norilsk industrial zone for metal mining and production [14]. The present work confirms the presence of spore-forming SRB in oxidized sediments. Their actual activity in acidic oxidized sediments of gold mine tailings of the Kuznetsk Basin requires further investigation.

The presence of aerobic *A. ferrooxidans* in anaerobic microcosms with active sulfate reduction also contradicts the accepted view on the physiology and geochemical role of this organism. *A. ferrooxidans* was shown to grow chemolithotrophically under anaerobic conditions, with Fe(III) as an electron acceptor [22]. The geochemical role of this process is not understood. The obtained evidence for development of "traditional" aerobes in anaerobic microcosms and of anaerobic microorganisms in oxidized acidic sediments confirms the necessity for the study of the activity of these microorganisms in "nontraditional" environments.

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REFERENCES

1. Blowes, D.W., Ptacek, C.J., and Weisener, C.G., The Geochemistry of Acid Mine Drainage, *Treatise on Geochemistry*, 2003, vol. 9, pp. 149–204.
2. Johnson, B., Biological Removal of Sulfurous Compounds from Inorganic Wastewaters, in *Environmental technologies to Treat Sulfur Pollution: Principles and Engineering*, Lens, P.N.L. and Hulshoff, L., Eds., London: IWA Publishing, 2000, pp. 175–205.
3. Gerashchenko, A.A., Analysis of the Mineral Source Base of Gold in the Kemerovo District, in *Zoloto Kuzbassa* (Gold of the Kuznetsk Basin), Kemerovo: Kemerovskii poligrafkombinat, 2000, pp. 69–213.
4. Widdel, F.F and Bak, R., Gram-Negative Mesophilic Sulfate-Reducing Bacteria, in *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, 2nd ed.*, Balows, A., et al., Eds., Berlin: Springer-Verlag, 1992, vol. 4, pp. 3352–3378.
5. Muyzer, G., Hottenträger, S., Teske, A., and Wawer, C., Denaturing Gradient Gel Electrophoresis of PCR-Amplified 16S rDNA–a New Molecular Approach to Analyze the Genetic Diversity of Mixed Microbial Communities, in *Molecular Microbial Ecology Manual*, Akkermans, A.D.L., et al., Eds., Dordrecht: Kluwer Academic Publishers, 1996, pp. 1–23.
6. DeLong, E.F., Archaea in Costal Marine Environments, *Proc. Natl. Acad. Sci. USA*, 1992, vol. 89, pp. 5685–5689.
7. Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J., 16S Ribosomal DNA Amplification for Phylogenetic Study, *J. Bacteriol.*, 1991, vol. 173, pp. 697–703.
8. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J., Gapped BLAST and PSI-BLAST: a New Generation of Protein Database Search Programs, *Nucleic Acids Res.*, 1997, vol. 25, pp. 3389–3402.
9. Hamamura, N., Olson, S.H., Ward, D.M., and Inskeep, W.P., Diversity and Functional Analysis of Bacterial Communities Associated with Natural Hydrocarbon Seeps in Acidic Soils at Rainbow Springs, Yellowstone National Park, *Appl. Environ. Microbiol.*, 2005, vol. 71, pp. 5943–5950.
10. Garcia-Moyano, A., González-Toril, E., Aguilera, A., and Amils, R., Prokaryotic Community Composition and Ecology of Floating Macroscopic Filaments from an Extreme Acidic Environment, Rio Tinto (SW, Spain), *Syst. Appl. Microbiol*, 2007, vol. 30, pp. 601–614.
11. Labrenz, M. and Banfield, J.F., Sulfate-Reducing Bacteria-Dominated Biofilms That Precipitate ZnS in a Subsurface Circumneutral-pH Mine Drainage System, *Microbial Ecol.*, 2004, vol. 47, pp. 205–217.
12. Dopson, M. and Lindström, E.B., Potential Role of *Thiobacillus caldus* in Arsenopyrite Bioleaching, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 36–40.
13. Stahl, D.A., Fishbain, S., Klein, M., Baker, B.J., and Wagner, M., Origins and Diversification of Sulfate-Respiring Microorganisms, *Antonie van Leeuwenhoek*, 2002, vol. 81, pp. 189–195.
14. Karnachuk, O.V., Pimenov, N.V., Yusupov, S.K., Frank, Y.A., Kaksonen, A.H., Puhakka, J.A., Ivanov, M.V., Lindström, E.B., and Tuovinen, O.H., Sulfate Reduction Potential in Sediments in the Norilsk Mining Area, Northern Siberia, *Geomicrobiol. J.*, 2005, vol. 22, pp. 11–25.
15. Johnson, B.D. and Hallberg, K.B., The Microbiology of Acidic Mine Waters, *Res. Microbiol*, 2003, vol. 154, pp. 466–473.
16. Nevin, K.P., Finneran, K.T., and Lovley, D.R., Microorganisms Associated with Uranium Bioremediation in a

High-Salinity Subsurface Sediment, *Appl. Environ. Microbiol.*, 2003, vol. 69, pp. 3672–3675.

17. Petrie, L., North, N.N., Dollhopf, S.L., Balkwill, D.L., and Kostka, J.E., Enumeration and Characterization of Iron(III)-Reducing Microbial Communities from Acidic Subsurface Sediments Contaminated with Uranium(VI), *Appl. Environ. Microbiol.*, 2003, vol. 69, pp. 7467–7479.
18. Saunders, J.A., Lee, M.-K., Wolf, L.W., Morton, C.M., Feng, Y., Thomson, I., and Park, S., Geochemical, Microbiological, and Geophysical Assessments of Anaerobic Immobilization of Heavy Metals, *Bioremediation J*, 2005, vol. 9, pp. 33–48.
19. Newman, D.K., Kennedy, E.K., Coates, J.D., Ahmann, D., Ellis, D.J., Lovley, D.R., and Morel, F.M.M., Dissimilatory Arsenate and Sulfate Reduction in *Desulfotomaculum auripigmentum* sp. nov, *Arch. Microbiol.*, 1997, vol. 168, pp. 380–388.
20. Sokolova, T.G., Kostrikina, N.A., Chernyh, N.A., Kolganova, T.V., Tourova, T.P., and Bonch-Osmolovskaya, E.A., *Thermincola carboxydiphila* gen. nov., sp. nov., a Novel Anaerobic, Carboxydotrophic, Hydrogenogenic Bacterium from a Hot Spring of the Lake Baikal Area, *Int. J. Syst. Evol. Microbiol*, 2005, vol. 55, pp. 2069–2073.
21. Zavarzina, D.G., Sokolova, T.G., Tourova, T.P., Chernyh, N.A., Kostrikina, N.A., and Bonch-Osmolovskaya, E.A., *Thermincola ferriacetica* sp. nov., a New Anaerobic, Thermophilic, Facultatively Chemolithoautotrophic Bacterium Capable of Dissimilatory Fe(III) Reduction, *Extremophiles*, 2007, vol. 11, pp. 1–7.
22. Ohmura, N., Sasaki, K., Matsumoto, N., and Saiki, H., Anaerobic Respiration Using Fe(3+), S(0), and H(2) in the Chemolithoautotrophic Bacterium *Acidithiobacillus ferrooxidans, J. Bacteriol.*, 2002, vol. 184, pp. 2081–2087.