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EXPERIMENTAL ARTICLES

Identification of the Dominant Bacterium of Two-Stage Biooxidation of Gold–Arsenic Concentrate

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Abstract—In the process of biooxidation at 39°C in a continuous mode of the gold–arsenic concentrate from the Olympiadinskoe deposit, which was pretreated by chemical leaching with ferric ions, by a microbial association from the BIO department reactors of the Polyus gold mining company, a bacterial culture designated as strain HT-4 was isolated. The bacterium was a spore-forming rod 0.5–0.6 × 1.4–2.0 μm with a flagellum. The optimal temperature for growth and Fe^{2+} oxidation was 55°C. The strain grew in the pH range from 1.21 to 2.10 with the optimum at pH 1.6. The organism was incapable of lithotrophic and organotrophic growth. It grew mixotrophically by Fe^{2+} oxidation in the presence of 0.02% yeast extract. The DNA G+C base content was 48.6 mol %. Based on comparative phylogenetic analysis of 1472-bp nucleotide sequences of 16S rRNA genes, strain HT-4 was classified as *Sulfobacillus thermosulfidooxidans*. Analysis by pulse-field gel electrophoresis revealed a unique profile of the *Not*I fragments of the chromosomal DNA. These results demonstrate the strain and species diversity of sulfobacilli in microbial associations involved in biooxidation of concentrates in different technological conditions. The strain "*S. olympiadicus* S-5" dominated in the process of biooxidation of original concentrate not treated with ferric iron, while *S. thermosulfidooxidans* HT-4 was predominant in biooxidation of the chemically leached concentrate.

Key words: biooxidation of gold–arsenic concentrate, *Sulfobacillus thermosulfidooxidans,* "*S. olympiadicus,*" cell morphology, growth rate, oxidation rate, organic substrates, mixotrophic growth, 16S rRNA gene analysis, *Not*I DNA fragment profile.

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Tank technology of bacterial oxidation of sulfide minerals from refractory gold–arsenic ores is the most efficient and environmentally safe of the known biohydrometallurgical technologies for recovery of noble metals. The technology ensures optimal conditions for the microorganisms.

Since 2001, a department for microbially mediated gold recovery has been in operation in Krasnoyarsk krai (Russia) at the Polyus gold mining company. Since the reactor temperature is maintained at 39–40°C, bacterial–chemical oxidation of the concentrates from pyrrhotite-containing pyrite–arsenopyrite gold–arsenic ore from the Olympiadinskoe deposit is carried out by a microbial association dominated by moderately thermophilic bacteria of the genus *Sulfobacillus*, including the new species "*S. olympiadicus*" [1]. Application of moderately thermophilic bacteria makes it possible to increase the process rate and decrease the expense of water and energy for reactor cooling at intense heating of the reaction mass due to exothermic reactions.

Duration of the process of tank bacterial oxidation of sulfide minerals in industrial bioreactors (up to 120 h) promoted development of more efficient technologies [2]. Short-term chemical leaching of the concentrates at 80°C by ferric ions formed in the liquid phase during bacterial oxidation of the concentrate was found to increase significantly both the rate and the extent of subsequent bacterial–chemical oxidation of the main sulfide minerals [3].

Microbial associations including various species and strains of *Sulfobacillus* are involved in bacterial–chemical oxidation of various types of sulfide concentrates [4]. Changes of the mineral composition of concentrate, of the ratio of minerals, or of the content of organic matter in the liquid phase (arriving with water) result in another member of the genetically heterogeneous population better adapted to the new conditions becoming the dominant strain. Since biogeotechnological processes are carried out under nonsterile conditions, autochthonous microorganisms of the deposit are responsible for the oxidation of sulfide minerals [5].

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Chemical leaching of the concentrate is carried out at 80°C. Such conditions do not rule out the presence of autochthonous sulfobacilli (forming heat-resistant spores) at the subsequent stage of bacterial oxidation. Isolation and identification of the dominant culture is therefore of significant interest for the new two-stage technology employing chemical leaching of the concentrate with ferric ions at the first stage and biooxidation at the second stage.

The goals of the present work were isolation of the dominant microbial culture responsible for biooxidation of chemically leached gold–arsenic concentrate of the Olympiadinskoe deposit ore inoculated with a microbial association from the pulp of the BIO department reactors of the Polyus gold mining company, investigation of its phenotypic and genotypic characteristics, and determination of its taxonomic status.

MATERIALS AND METHODS

The object of research was the microbial association from the pulp of the BIO department reactors of the Polyus gold mining company. The association included the strains "*Sulfobacillus olympiadicus* S-5," *Ferroplasma acidiphilum* Y-9 and Y-10, and *Leptospirillum ferrooxidans* L-5; small amounts of *Acidithiobacillus ferrooxidans* TFO and *A. thiooxidans* TTO; and the fungus *Aspergillus niger* [1]. Strain "*S. olympiadicus* S-5" predominated in the association (60–80% of the total cell number).

Oxidation of the gold–arsenic concentrate treated with ferric ions at 80°C was carried out by the microbial association in two 2.5-l reactors with 1 l of 9KS mineral medium [6] and 150 g/l of the concentrate in the feed–batch mode imitating a continuous process (with removal of 50% of the pulp and 12-h cycle duration) with mixing by a turbine stirrer at 430 rpm. The temperature (39°C) was maintained by heat exchangers connected to a TW2.02 ultrathermostat (Elmi, Latvia). Air was supplied by a microcompressor (4 l/min). The inoculum ratio was 10% (vol/vol).

Isolation of pure microbial cultures and cultivation conditions. Pure cultures were obtained by inoculating tenfold dilutions of the liquid phase of the pulp into selective media at 39°C. Modified 9KS medium [6] supplemented with 0.02% yeast extract and the same medium with elemental sulfur as an energy source instead of $FeSO_4 \cdot 7H_2O$ were used for dilutions. Pure cultures were obtained by subsequent transfers of tenfold dilutions. Purity of the cultures was ascertained by the absence of growth in liquid medium containing 0.15 g yeast extract, 0.15 g peptone, or 0.055 g sodium pyruvate. These components were added to 9KS medium without ferrous iron (pH was adjusted to 2.2 with 10 N H_2SO_4).

The strains were maintained on 9KS medium supplemented with 0.02% yeast extract and 1 mM $Na_2S_2O_3$. The cultivation was carried out on a rotary shaker (170 rpm) in 250-ml Erlenmeyer flasks with 100 ml of the medium and 10 ml of inoculum.

Electron microscopy. For total cell preparations, the cells were concentrated by centrifugation at 4600 *g* for 3 min. Cells grown on ferrous iron or sulfide concentrates were contrasted by the iron ions of the medium, so additional staining was not required. The samples were investigated in a JEM-100C electron microscope (Japan).

Phenotypic characterization. The effect of temperature and pH on microbial growth was determined on 9KS medium supplemented with 0.02% yeast extract and 1 mM $Na_2S_2O_3$. Capacity for lithotrophic growth was assayed by cultivation on 9KS medium without organic compounds. Capacity for growth on organic substrates was determined by inoculation of 9KS medium without ferrous iron supplemented with one of the following organic compounds (0.02%): fructose, sucrose, glycine, methionine, glucose, citrate, acetate, succinate, glutathione, and yeast extract. Capacity for mixotrophic growth was determined on 9KS medium supplemented with the same organic compounds (0.02%). Capacity for growth under mixotrophic conditions was confirmed by several transfers. Organic compounds were filter-sterilized (0.2 μm). The pH value was adjusted with 10 N H_2SO_4 to 2.5 for growth on S^0, 1.9–2.0 for growth on organic substrates and 1.7–1.8 for growth on Fe^{2+}.

Analysis of DNA structure. DNA from bacterial cells was isolated by the Marmur method [7]. The nucleotide composition was determined by heat denaturation [8]. Precipitation and washing of the biomass, as well as isolation of the native chromosomal DNA, were carried out according to [9]. The structure of the chromosomal DNA was analyzed by pulse-field gel electrophoresis of the fragments cleaved with *Not*I restriction endonuclease (30 U/30 μl) with the GC↓GGCCGC sequence at the restriction site [9].

16S rRNA gene sequencing. Amplification and sequencing of 16S rRNA genes were carried out with the primers universal for most prokaryotes: 11f (5'-AGAGTTTGATCMTGGCTCAG-3'), 519r (5'-GWATTACCGCGGCKGCTG-3'), 530f (5'-GTGCCAGCMGCCGCGG-3'), 1114f (5'-GCAACGAGCGCAACCC-3'), and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') [10]. Amplification was carried out of a Cetus 480 device (Perkin Elmer, Sweden) with heat-stable Bio*Taq* DNA polymerase (Dialat Ltd., Russia) according to the manufacturer's recommendations. Amplification (30 cycles) was carried out as follows: denaturation at 94°C, 30 s; primer annealing at 40°C, 1 min; and elongation at 72°C, 2 min 30 s. The PCR products were analyzed by electrophoresis in 1% agarose gel. After purification on low-melt agarose and on columns (Promega, United States), sequencing of 1472 nucleotides of 16S rRNA genes was carried out with a Silver Sequencing kit (Promega, United States) according to the manufacturer's recommendations. The 16S rRNA gene sequence of strain HT-4

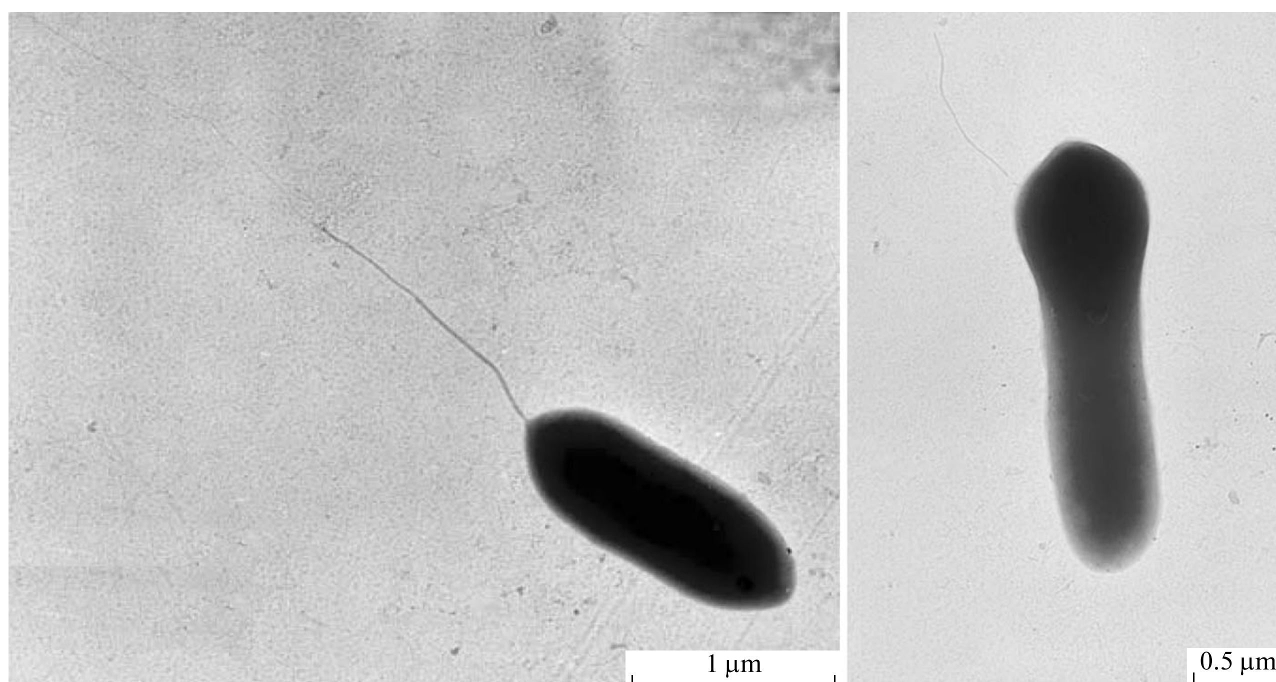

Fig. 1. Cell morphology of strain HT-4. Flagellated cell (left) and a cell with the prospore (right).

was deposited in GenBank under accession no. GU180244. The unrooted phylogenetic tree was constructed by the methods implemented in the TREECON software package [11].

RESULTS

After chemical leaching of the Olympiadinskoe deposit ore concentrate with ferric ions at 80°C, the dominant bacterial culture designated as strain HT-4 was isolated by tenfold dilutions from the microbial community of the liquid phase of the pulp during biooxidation at 39°C.

The cell morphology of strain HT-4 is shown in Fig. 1. The cells are rods of 0.5–0.6 × 1.4–2.0 μm possessing a flagellum and forming spores.

Table 1 presents the kinetic parameters of strain HT-4 growth on medium with Fe^{2+} at different temperature values. It can be seen that 55°C is the optimal temperature, resulting in the highest growth rate, rate of ferrous iron oxidation, and biomass density.

Table 2 demonstrates the effect of initial pH on growth of strain HT-4 at 55°C. The highest values of the rates of growth and ferrous iron oxidation, as well as the shortest generation time were observed at pH 1.60.

Under autotrophic conditions, strain HT-4 grew only in the first transfer due to utilization of intracellular reserves.

Utilization of organic compounds by strain HT-4 is presented in Table 3. The number of transfers varied depending on the parameters of cell growth. It can be seen that strain HT-4 was almost incapable of organotrophic growth. By the third transfer, no growth occurred on most substrates; although weak growth was observed on fructose and yeast extract, it was doubtlessly diminishing.

Mixotrophic growth of strain HT-4 was studied by 48-h incubation in medium with ferrous iron and various organic compounds. Only in the presence of yeast extract (0.02%) were optimal growth conditions obtained. Fructose and citrate supported very weak growth in the third transfer.

Table 1. Kinetic growth parameters for strain HT-4 on medium with Fe^{2+} at different temperatures and initial pH 1.9

Temperature, °C	35	40	45	50	55	60
Specific growth rate, h^{-1}	0.025	0.035	0.14	0.15	0.39	0.16
Generation time, h	27.72	19.80	4.95	4.62	1.77	4.33
Cell number per 1 ml, $\times 10^7$	0.099	1.93	3.6	3.7	7.05	4.31
Rate of Fe^{2+} oxidation, g l^{-1} h^{-1}	0.14	0.28	0.32	0.60	0.66	0.48

Table 2. Effect of initial pH value on the kinetic parameters of strain HT-4 growth at 55°C

Initial pH value	Maximal specific growth rate, h^{-1}	Generation time, h	Rate of Fe^{2+} oxidation, $g\ l^{-1}\ h^{-1}$
1.21	0.28	2.48	0.17
1.40	0.36	1.95	0.20
1.49	0.68	1.02	0.23
1.60	0.75	0.92	0.41
1.80	0.61	1.14	0.25
2.00	0.48	1.44	0.19
2.10	0.30	2.31	0.17

Active oxidation of sulfide minerals of the concentrates containing metal ions and sulfide sulfur requires oxidation of both components. In bacterial associations containing different strains of *Sulfobacillus*, strains are usually present that preferentially oxidize either of the components. Since strain HT-4 was the only dominant strain at the stage of biooxidation of the concentrate after chemical leaching with ferric ions, it should be expected to oxidize reduced sulfur compounds. Investigation of capacity of strain HT-4 for oxidation of elemental sulfur, thiosulfate, and tetrathionate confirmed this suggestion. On the third day of cultivation with elemental sulfur, thiosulfate, or tetrathionate, the cell concentrations were 2.06×10^8, 8.85×10^7, and 2.12×10^8 cell/ml, respectively. During this process, pH values decreased from 2.2 to 1.9, 2.02, and 2.03, respectively.

These results suggested classification of strain HT-4 as an acidophilic, chemolithotrophic, and moderately thermophilic spore-forming bacterium with mixotrophic metabolism within the genus *Sulfobacillus*.

Its DNA G+C base content (48.6 mol %) is close to the values for four out of the six presently known *Sulfobacillus* species, *S. thermosulfidooxidans, S. sibiricus, S. thermotolerans*, and "*S. olympiadicus*" (Table 5).

Comparison of 16S rRNA gene sequences of strain HT-4 with the sequences of other *S. thermosulfidooxidans* strains (Fig. 2) revealed high homology (98.8–99.9%), as well as 99.9% homology with the type strain. The similarity between strain HT-4 and other *Sulfobacillus* species, apart from *S. thermosulfidooxidans*, was significantly lower (90.0–98.5%). These data make it possible to classify strain HT-4 within the species *S. thermosulfidooxidans*.

The profiles of *Not*I chromosomal DNA fragments analyzed by pulse-field gel electrophoreses for three strains of *Sulfobacillus*—namely, *S. thermosulfidooxidans* HT-1, *S. thermosulfidooxidans* HT-4, and "*S. olympiadicus*" S-5—are presented on Fig. 3. The first strain was isolated from reactor pulp in the traditional one-stage process of biooxidation of the Olympiadinskoe ore concentrate at 50°C (unpublished data). Strain S-5 predominates in the microbial association involved in biooxidation of the Olympiadinskoe ore concentrate at 39°C in the BIO department reactors of the Polyus gold mining company. Strain HT-4 was isolated from the two-stage process of oxidation of the same concentrate at 39°C after leaching at 80°C. Different strains of *Sulfobacillus* predominated in the processes at different temperatures or substrate characteristics. All strains have a unique chromosomal DNA structure. This is used for identification of the strains predominant in biohydrometallurgical technologies and for analysis of the strain composition of microbial communities in the

Table 3. Growth of strain HT-4 on medium with organic compounds as carbon and energy sources at 55°C (incubation time, 48 h)

Organic compound	Cell number in the 1st transfer, $\times 10^7$	Cell number in the 2nd transfer, $\times 10^7$	Cell number in the 3rd transfer, $\times 10^7$
Glutathione	0.93	Single cells	No
Succinate	Single cells	No	No
Acetate	0.22	No	No
Citrate	0.34	No	No
Glucose	0.34	0.20	No
Glycine	0.09	No	No
Sucrose	Single cells	No	No
Fructose	1.53	1.28	0.39
Methionine	0.28	No	No
Yeast extract	0.73	0.13	0.12

Table 4. Mixotrophic growth of strain HT-4 on medium with ferrous iron and organic compounds at 55°C

Organic compound	Cell number in the 1st transfer, $\times 10^7$	Cell number in the 2nd transfer, $\times 10^7$	Cell number in the 3rd transfer, $\times 10^7$
Glutathione	6.15	1.75	0.89
Succinate	1.78	0.39	No
Acetate	1.89	No	No
Citrate	0.89	0.68	0.27
Glucose	1.20	0.46	No
Glycine	1.78	0.27	No
Sucrose	1.72	1.29	No
Fructose	1.23	0.53	0.39
Methionine	0.58	0.29	No
Yeast extract	5.80	6.0	5.90

monitoring of biooxidation of sulfide minerals of gold–arsenic concentrates under permanently varying conditions (characterization of the energy substrate, temperature, pH, presence of organic compounds, etc.)

Information concerning the profiles of the *Not*I chromosomal DNA fragments for the known *Sulfobacillus* species [13] made it possible to determine that strain HT-4 has been previously unknown. In order to determine the taxonomic status of the strain, its phenotypic and genotypic characteristics were analyzed and the strain was classified within the species *S. thermosulfidooxidans*. It was demonstrated that, in the process of bacterial–chemical oxidation of the gold–arsenic concentrate of the Olympiadinskoe deposit chemically leached with ferric ions in a continuous mode at 39°C, the inoculated strain "*S. olympiadicus*" S-5 (dominating in the biooxidation of the original concentrate) was replaced by strain *S. thermosulfidooxidans* HT-4.

Table 5. DNA G+C base content of type strains of different *Sulfobacillus* species [11]

Sulfobacillus species	G+C content, mol %
S. thermosulfidooxidans 1269^T	48–50
S. thermosulfidooxidans subsp. *asporogenes* 41^T	45.5
S. acidophilus NAL^T	55–57
S. sibiricus N1^T	48–50
S. thermotolerans Kr1^T	48.2
"*S. olympiadicus*" S-5	48.9
S. benefaciens BRGM2^T	50.6

DISCUSSION

Associations of acidophilic chemolithotrophic microorganisms obtaining energy via oxidation of sulfide minerals, ferrous ions, elemental sulfur, or reduced sulfur compounds are formed in sulfide ore deposits and in the technological processes of bacterial–chemical oxidation of ore concentrates. The species composition of microbial associations depends primarily on the temperature conditions of the biooxidation process. The same sulfide concentrate is oxidized by one microbial association under mesophilic conditions (28–30°C), by another under moderately thermophilic conditions (40–50°C), and by yet another one under thermophilic conditions (over 60°C). For example, under mesophilic conditions, oxidation of the gold–arsenic concentrate from the pyrrhotite-containing pyrite–arsenopyrite ore of the Olympiadinskoe deposit is carried out mainly by *A. ferrooxidans* and *A. thiooxidans*. Under moderately thermophilic conditions, strains of gram-positive *Sulfobacillus* predominate with participation of gram-negative *L. ferrooxidans* and archaeal *F. acidiphilum*, while *A. ferrooxidans, A. thiooxidans*, and *Aspergillus niger* are always present in the pulp [1]. The species composition of microbial associations is not highly diverse. However, high polymorphism of the strains of acidophilic chemolithotrophic microorganisms should be mentioned. Depending on the characteris-

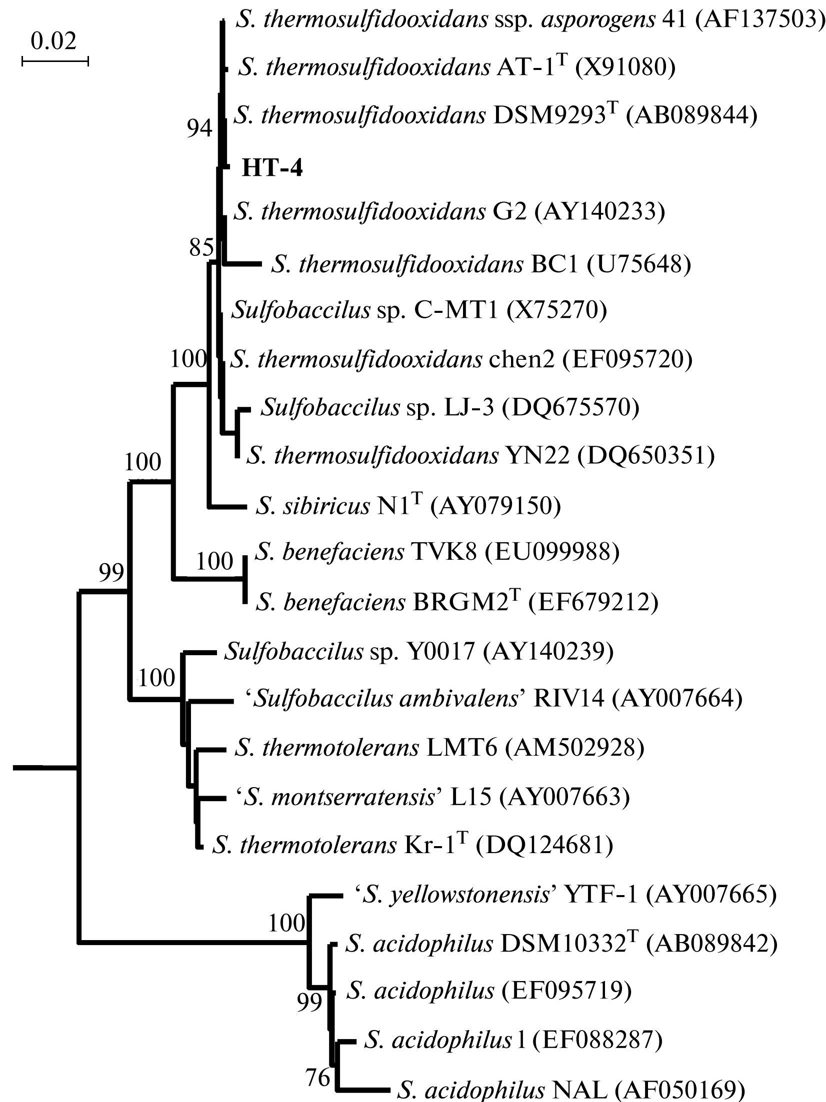

Fig. 2. Phylogenetic tree based on comparison of 16S rRNA gene sequences demonstrating the position of strain HT-4. Scale, two nucleotide replacements per 100 nucleotides. The numbers indicate bootstrap confidence values (bootstrap values above 75 were accepted as significant).

tics of the oxidized substrates, strain composition of a microbial association may vary, while the species composition remains constant [5].

Extensive monitoring of the microbial association of the pulp from the reactors of the BIO department of the Polyus gold mining company revealed a continuous process of replacement of the dominant strains of *Sulfobacillus* by other ones. In the present work, it was demonstrated that changes in the characteristics of the concentrate resulting from its high-temperature treatment with ferric ions also resulted in the change of the dominant *Sulfobacillus* strain. Apart from strain heterogeneity within a species of *Sulfobacillus*, species heterogeneity was also revealed in the process of sulfide ore oxidation. In microbial associations of the pulps of the concentrates oxidized according to the traditional technology at 39°C, "*S. olympiadicus*" was the dominant microorganism. When the concentrate was chemically leached by ferric ions at 80°C prior to biooxidation at 39°C, strain *S. thermosulfidooxidans* HT-4 was the most successful one.

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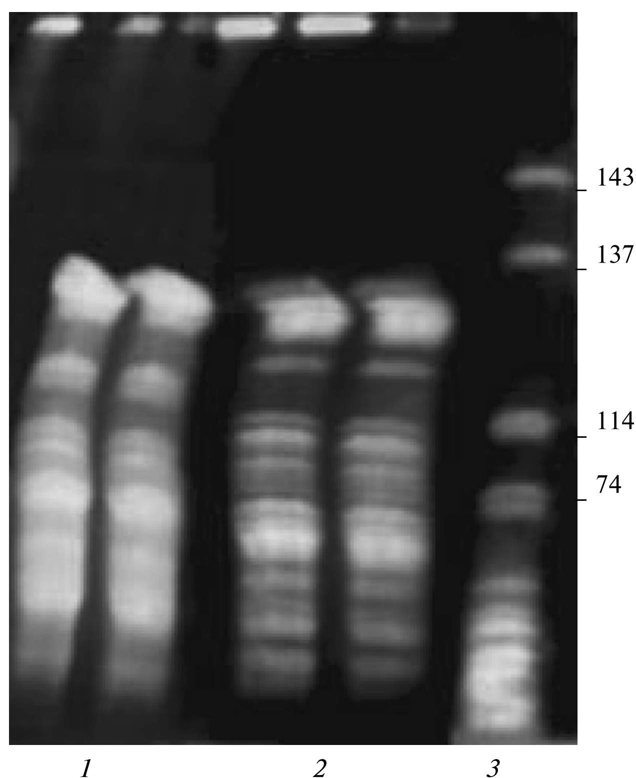

Fig. 3. Profiles of the *Not*I fragments of the chromosomal DNA of *Sulfobacillus* strains isolated from the pulp during bacterial-chemical oxidation of the Olympiadinskoe deposit ore concentrate. Strains: HT-1 (*1*), HT-4 (*2*), and S-5 (*3*). Pulse-field electrophoresis conditions: 13 V/cm, 10°C, 10-s pulse duration, total duration 65 h. DNA fragment sizes (kb) are indicated on the right.

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