ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 3, pp. 326–334. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © A.G. Bulaev, T.A. Pivovarova, V.S. Melamud, I.A. Tsaplina, A.E. Zhuravleva, T.F. Kondrat'eva, 2011, published in Mikrobiologiya, 2011, Vol. 80, No. 3, pp. 320–328.

EXPERIMENTAL ARTICLES

Polymorphism of *Sulfobacillus thermosulfidooxidans* Strains Dominating in Processes of High-Temperature Oxidation of Gold–Arsenic Concentrate

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Received November 3, 2010

Abstract—The composition was studied of the microbial association involved in tank biooxidation of the concentrate of a refractory pyrrhotite-containing pyrite–arsenopyrite gold–arsenic ore from the Olympiadinskoe deposit at 50°C. The two *Sulfobacillus thermosulfidooxidans* strains predominant in the association were phylogenetically different from the strains used as inocula. The isolates were found to differ significantly both from each other and from the strains that dominated in the processes of biooxidation of a similar concentrate by traditional tank technology at 39°C or at 39°C with treatment of the concentrate with ferric iron prior to biooxidation. These results indicate the strain and species diversity of sulfobacilli in microbial associations involved in biooxidation of the concentrates under different technological modes.

Keywords: tank biooxidation of sulfide ores, chemolithotrophic acidophilic associations, moderate thermophiles, *Sulfobacillus thermosulfidooxidans*, strain polymorphism, pulse-field gel electrophoresis, PCR.

DOI: 10.1134/S0026261711030052

Bacteria of the genus *Sulfobacillus* are moderately thermophilic gram-positive chemolithotrophic microorganisms capable of oxidation of ferrous iron, elemental sulfur, reduced sulfur compounds, and sulfide minerals, which commonly occur in sulfide ore deposits. They were isolated and described as an independent genus in the 1970s [1, 2]. Several species of this genus are presently known: *S. thermosulfidooxidans, S. acidophilus, S. sibiricus, S. thermotolerans, S. olympiadicus'*, and *S. benefaciens* [3–7]. The oxidative activity of these microorganisms was shown to cause a significant heating of sulfide ores. Members of this genus may play a significant part in industrial biohydrometallurgical techniques for the oxidation of sulfide minerals, since oxidation is more intense at elevated temperatures than under mesophilic conditions [8]. Moderately thermophilic bacteria have been initially used in laboratory trials on biooxidation of the gold–arsenic ore concentrate in the Institute of Microbiology, Russian Academy of Sciences, in 1999 [9]. The trials demonstrated that biooxidation by sulfobacilli at elevated temperatures was more efficient than in the case of mesophilic microorganisms.

Sulfobacilli are known to be used in industrial processes of sulfide ore biooxidation. They were found in the processes of heap bioleaching of copper in the Monywa deposit (Australia) [10], oxidation of Agnes gold-containing ore (South Africa) [11], and in the pulp of the reactors for bioleaching of cobalt-containing pyrite (Kasese, Uganda) [12]. Application of bacteria closely related to *S. thermosulfidooxidans* for tank bacterial gold recovery at 45–55°C at the Youanmi facility (Australia) was reported [13].

In Russia, the gold-recovering factory (GRF) of the Polyus Corp. that has functioned since 2001 uses bacterial–chemical oxidation of concentrates from the pyrrhotite-containing pyrite–arsenopyrite gold–arsenic ore of the Olympiadinskoe deposit according to the BIONORD technology [14]. At a 39–40°C temperature in the reactors, moderately thermophilic *Sulfobacillus* species, including the new species *S. olympiadicus*, play the dominant role in this process [15]. Different species and strains of *Sulfobacillus* are present in microbial associations participating in the oxidation of different types of sulfide ore concentrates during bacterial–chemical processes [16].

Duration of tank bacterial oxidation in industrial reactors (up to 120 h) and low degree of oxidation of such concentrate components as pyrite and antimonite at 39–40°C promoted development of new technologies. A two-stage scheme is one of the pathways to increased efficiency [17]. Monitoring of the composition of microbial associations during two-stage biooxidation of the flotation concentrate of sulfide gold–arsenic ore at 39°C revealed that the microorganisms used as inocula were displaced by new strains of sulfobacilli. One of the dominant microorganisms was identified and described earlier [18].

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Increasing the operating temperature to 50°C and application of the association of *Sulfobacillus* strains adapted to high temperature is another way to increase the efficiency of the process [19, 20]. Moreover, operation at elevated temperatures makes it possible to decrease the cost of cooling of industrial reactors. Biooxidation of sulfide minerals is an exothermic process, so the temperature rises to the values incompatible with activity of the mesophiles.

The goal of the present work was to describe the characteristics of the *Sulfobacillus* strains dominating during the oxidation of the gold–arsenic concentrate at 50°C and to compare them to the strains isolated during the trials of the two-stage technology and from the pulp of the reactors of the operating Olympiadinskoe GRF.

MATERIALS AND METHODS

Subjects of investigation. The microbial association from the pulp of the Polyus GRF reactors [14, 15] was used as an inoculum. It contained the strains of *S. olympiadicus*, *Leptospirillum ferrooxidans, Ferroplasma acidiphilum, A. ferrooxidans*, and *A. thiooxidans*; the fungus *Aspergillus niger*; the type strains *S. thermosulfidooxidans* 1269^T and *S. sibiricus* $N1^T$ [2, 3]; and the strains *S. sibiricus* B1, B2, B3, OFO, and SSO, which have been isolated from the ore self-heating zone of the Olympiadinskoe deposit [20]. The microorganisms of the association responsible for biooxidation of the ore concentrate at 50°C and the *S. thermosulfidooxidans* strain isolated from the reactor pulp of the Olympiadinskoe GRF were the subjects of investigation. In the present work, more complete description of the characteristics of previously described strain *S. thermosulfidooxidans* HT-4 [18] was carried out.

Isolation of pure cultures and cultivation conditions. Pure microbial cultures were obtained by inoculation of tenfold dilutions of the pulp in selective media at 50°C. The modified 9KS medium [21] supplemented with 0.02% of yeast extract (YE) and the medium of the same mineral composition with elemental sulfur (10 g/l) instead of $F2SO_4 \cdot 7H_2O$ were used for dilutions. Media with elemental sulfur were supplemented with 2 ml/l of the trace element solution containing the following (g/l): $FeCl_3 \cdot 6H_2O$, 1.1; $CuSO_4 \cdot 5H_2O$, 0.05; H_3BO_3, 0.2; $MnSO_4 \cdot H_2O$, 0.2; $NaMoO_4 \cdot 2H_2O$, 0.08; $CoCl_2 \cdot 6H_2O$, 0.06; and $ZnSO_4 \cdot 7H_2O$, 0.09. Pure cultures were obtained by repeated inoculations by the terminal dilution method. The absence of growth in liquid 9K medium with yeast extract (15 g/l), peptone (15 g/l), or sodium pyruvate (5.5 g/l) instead of ferrous iron was used to ascertain the purity of the cultures.

The microorganisms were grown at 50°C on a shaker (170 rpm) in 250-ml Erlenmeyer flasks with 100 ml of the medium. The inoculum volume was 10 ml.

The strains were maintained in 9KS medium with yeast extract (0.02%) and sodium thiosulfate (1 mM).

Light microscopy. Bacterial cells were enumerated under an Amplival phase contrast microscope (Carl Zeiss, Germany) in 20 fields of view.

Electron microscopy. To obtain the total cell preparations, the culture was centrifuged for 3 min at 4600 *g*. The cells grown with ferrous iron or sulfide concentrates were self-contrasted with ferric iron and therefore did not require additional contrasting. The samples were investigated under a JEM-100C electron microscope (Japan).

Physiological characteristics. The effects of temperature and pH on microbial growth were investigated in 9KS medium with yeast extract (0.02%) and sodium thiosulfate (1 mM).

Capacity for autotrophic growth was determined in 9KS medium without organic compounds. The capacity for growth on organic compounds was determined by inoculation of the mineral base of 9KS medium without ferrous iron, supplemented with one of the following compounds (0.05%): glucose, fructose, sucrose, glycine, methionine, citrate, acetate, succinate, glutathione, and yeast extract. The capacity for mixotrophic growth was determined in 9KS medium with 0.02% of the same organic compounds. Growth under different conditions was determined after several transfers. For growth on S_0, organic compounds, and Fe^{2+}, pH was adjusted with 10 N H_2SO_4 to 2.5, 1.9–2.0, and 1.7–1.8, respectively. The concentrations of ferric and ferrous iron were determined by chelatometric titration [22].

Analysis of the phylogenetic position by PCR and 16S rRNA gene sequencing. Amplification and sequencing of 16S rRNA genes was carried out using the universal prokaryotic primers [23]. Amplification was carried out using Cetus 480 (Perkin Elmer, Sweden) with the Bio Taq thermostable DNA polymerase (Dialat Ltd., Moscow, Russia) according to the manufacturer's recommendations. Isolation and purification of the PCR products from low-melt agarose were carried out with the Wizard PCR Prep kit (Promega, United States) according to the manufacturer's recommendations. After purification, 16S rRNA gene fragments were sequenced using the Silver Sequencing kit (Promega, United States) according to the manufacturer's recommendations. The unrooted phylogenetic tree was constructed using the TREECON software package [24].

Analysis of the structure of chromosomal DNA. The structure of the chromosomal DNA was determined by pulse-field gel electrophoresis of the fragments of the chromosomal DNA cleaved by the Not_1 restriction endonuclease as described earlier [25].

The values presented are averages of the results of three repeats in three independent experiments.

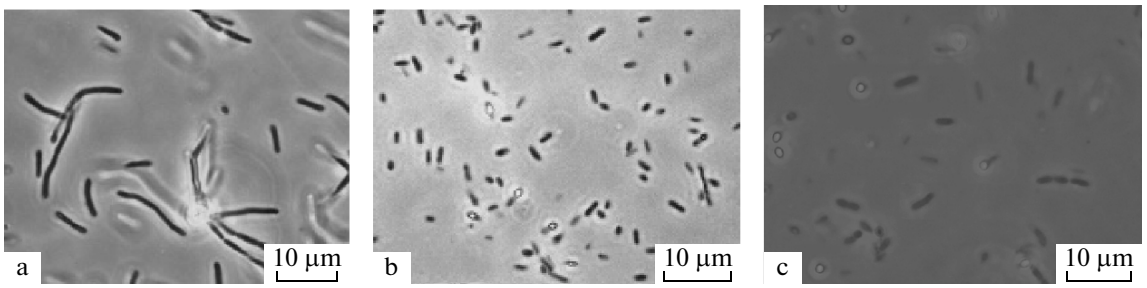

Fig. 1. Light microscopy of *Sulfobacillus* sp. cells, phase contrast: HT-1 (a), HT-3 (b), and HT-5 (c).

RESULTS AND DISCUSSION

Isolation of Pure Microbial Cultures

By inoculating tenfold dilutions of the liquid phase from the pulp of the reactor of a laboratory setup for bacterial–chemical oxidation of the gold–arsenic concentrate in selective media at 50°C, two isolates were obtained. The first, designated *Sulfobacillus* sp. HT-1, was isolated in 9KS medium with ferrous iron. The second, designated *Sulfobacillus* sp. HT-3, was isolated on the same medium with elemental sulfur instead of ferrous iron. The isolate designated *Sulfobacillus* sp. HT-5 was obtained from the pulp of the Olympiadinskoe GRF reactor in the medium with iron. These three strains were compared to the previously isolated strain *Sulfobacillus* sp. HT-4, which was involved in two-stage bacterial–chemical oxidation of the same concentrate at 39°C, when the stage of chemical oxidation with ferric iron was introduced prior to biooxidation.

Morphology of Microorganisms

In the exponential growth phase, the cells were rods with rounded ends, single or in short or long chains (Fig. 1). The cell sizes of different strains varied (Table 1). All strains exhibited polymorphism. Apart from HT-1, all isolates were found to form spores under experimental conditions. The spores were usually oval, sometimes spherical, terminally located, and somewhat expanding the sporangium (Fig. 1b). Although motility was not observed, electron microscopy revealed flagella in the isolates HT-1 and HT-5 (Figs. 2a, 2b).

Physiological Characteristics

Optimal values of pH and temperature. The specific growth rates depending on the cultivation temperature and pH are shown on Figs. 3 and 4, respectively. The greatest biomass yield and the highest rate of substrate oxidation occurred under optimal conditions. The data presented on Figs. 3 and 4 and in Table 2 demonstrate significant differences between the optimal temperature and pH values for different strains. Since strain HT-3 exhibited stable growth both on iron and elemental sulfur (see "oxidation of elemental sulfur"), the optimal growth parameters for this strain were determined on both substrates.

Oxidation of ferrous iron. Under mixotrophic conditions, all strains could oxidize iron at high rates. Transfer of the sulfur-oxidizing strain HT-3 to the medium with ferrous iron resulted in a length lag period of 24–30 h, after which all the iron in the medium was oxidized at a high rate. No lag period was observed after two transfers to the medium with ferrous iron. Reverse transfer to the medium with sulfur did not result in a lag phase.

Oxidation of elemental sulfur. While all the strains studied could oxidize elemental sulfur, none of them

Table 1. Morphological characteristics of *Sulfobacillus* sp. strains

Strain	Size, μm	Flagellation	Endospore formation
HT-1	1.3–1.8 × 1.5–6.0	Polar	Not observed
HT-3	0.5–1.0 × 0.3–2.0	Absent	+
HT-4	0.5–0.6 × 1.4–2.0	Monotrichous	+
HT-5	0.5–1.0 × 1.0–4.0	Peritrichous	+

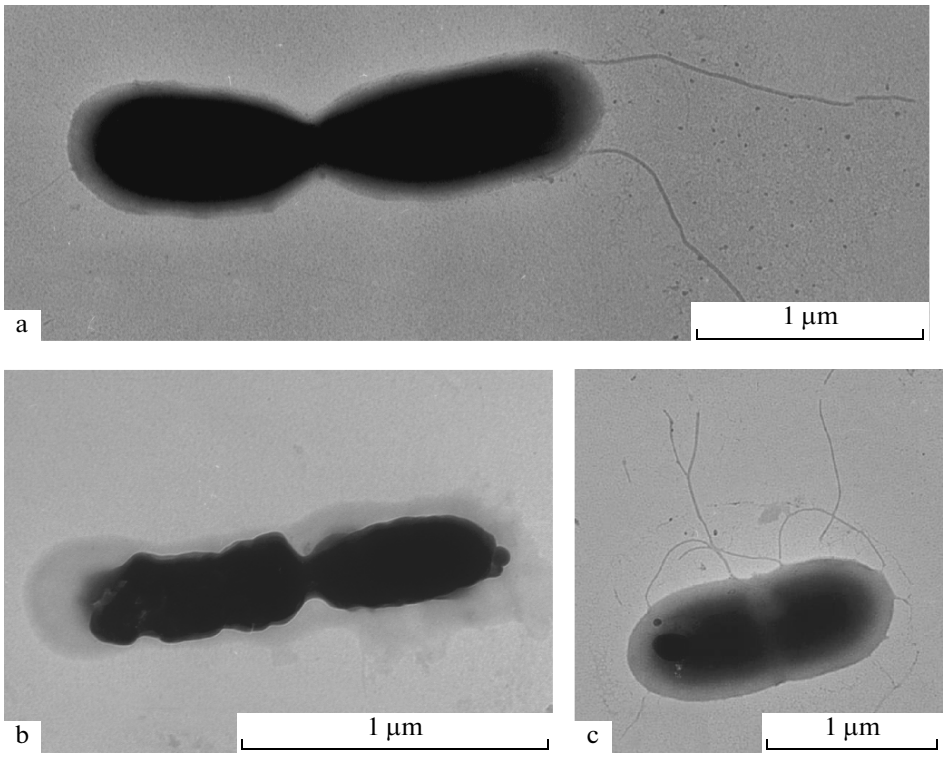

Fig. 2. Electron microscopy of *Sulfobacillus* sp. cells: HT-1 (a), HT-3 (b), and HT-5 (c).

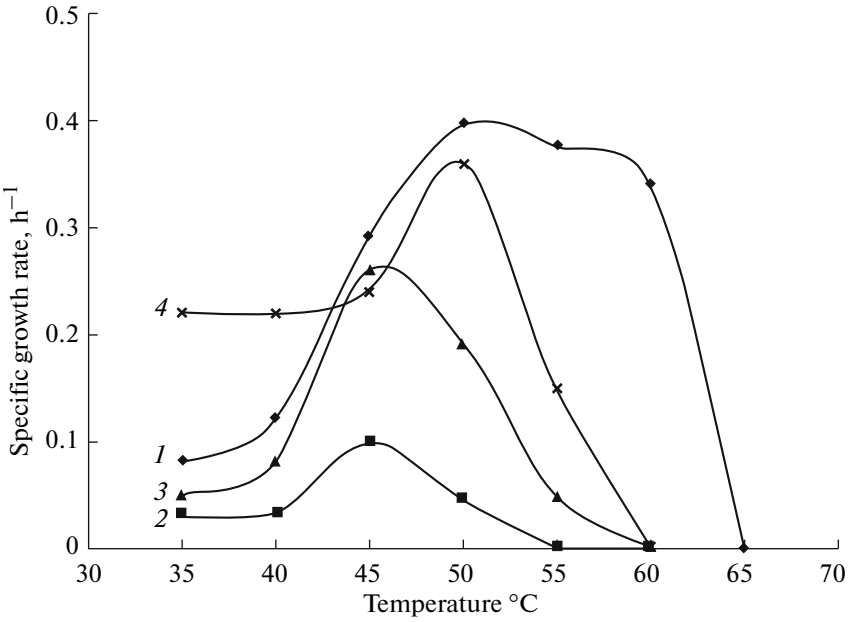

Fig. 3. Specific growth rate of *Sulfobacillus* sp. strains depending on temperature: HT-1 in the medium with iron (*1*), HT-3 in the medium with sulfur (*2*), HT-3 in the medium with iron (*3*), and HT-5 in the medium with iron (*4*).

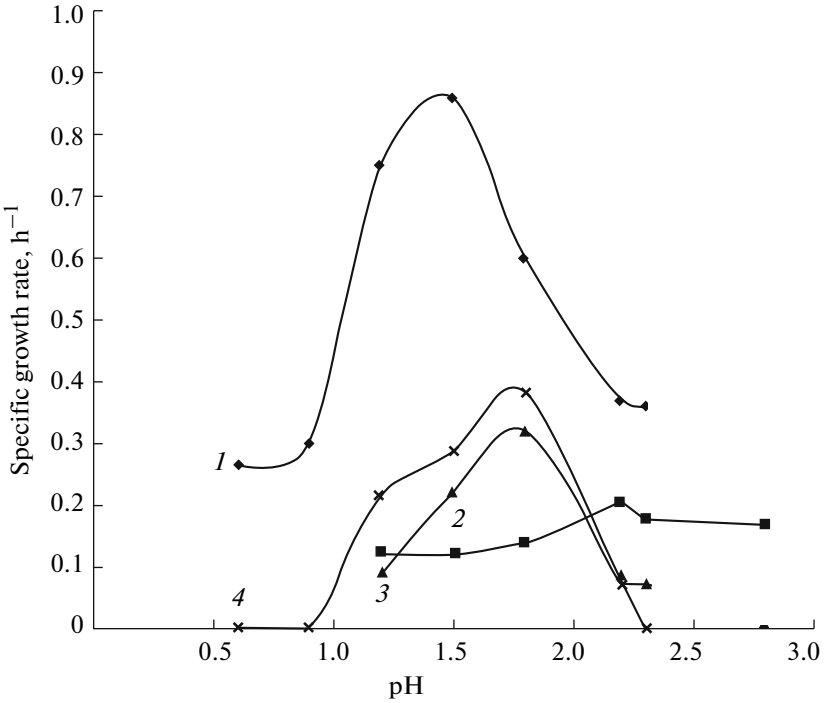

Fig. 4. Specific growth rate of *Sulfobacillus* sp. strains depending on pH: HT-1 in the medium with iron (*1*), HT-3 in the medium with sulfur (*2*), HT-3 in the medium with iron (*3*), and HT-5 in the medium with iron (*4*).

was able to oxidize sulfur without an organic carbon source. Apart from HT-3, which was isolated on the medium with elemental sulfur, all the isolates were able to oxidize sulfur only for a limited number of transfers (Table 3). In the remaining strains, the rate of sulfur oxidation decreased from transfer to transfer. The rate of sulfur oxidation was assessed by acidification of the medium.

Organic matter requirements. Apart from HT-5, all strains could grow in the medium without carbon sources for several transfers only (Table 4). For all strains, autotrophic growth on 9KS medium resulted in decreased rates of iron oxidation (complete oxidation of iron in 9KS medium required 9–12 h under mixotrophic conditions and 96–120 h under autotrophic ones) and decreased cell numbers/ml (from 10^8 under optimal conditions to 10^7). All strains could grow in a limited number of transfers in the medium with 0.05% YE as the sole energy source (Table 4). When the organic compounds listed in the Materials and Methods section were used as the sole energy sources (i.e., the medium did not contain YE and inorganic electron donors), the cultures could grow for no more than three transfers. Stable growth for ten or more transfers occurred only in the 9KS medium with iron and 0.02% of glucose, fructose, sucrose, methionine, or glutathione. Growth with other organic compounds was possible for not more than four transfers. The rate of iron oxidation in the media with all these compounds was, however, significantly lower than on media with YE. Complete oxidation of iron in 9KS medium with YE and with pure organic compounds took 9–12 and 48–72 h, respectively. The cell yield decreased from 10^8 to 10^7 cells/ml. Other organic compounds (glycine, citrate, acetate, and succinate) supported growth only for two to five transfers.

Table 2. Optimal temperature and pH values

Sulfobacillus sp. strain	*T*°C, optimum (upper range)	pH, optimum (ranges)
HT-1	50(65)	1.4–1.5 (0.5–2.8)
HT-3	45(55)	1.7 (1.2–2.8)
HT-4	55(@н.д.)	1.6 (1.2–2.8)
HT-5	50(55)	1.8 (1.2–2.4)

Genetic Characteristics

Analysis of the phylogenetic position of the microorganisms by PCR and 16S rRNA gene sequencing. Comparison of the nucleotide sequences of 16S rRNA

Table 3. Oxidation of elemental sulfur by *Sulfobacillus* sp. strains

Strain	Number of transfers for which sulfur oxidation was observed	Medium pH*/cell number ($\times 10^7$/ml) after 3-day incubation at optimal temperature			
		Transfer			
		1	2	3	4
HT-1	2	1.50/4.00	1.66/<1.00	2.15/<0.10	–
HT-3	Unlimited number of transfers	1.60/5.00	1.60/5.00	1.60/5.00	1.60/5.00
HT-4	3	1.75/2.50	1.36/5.00	1.70/<0.10	2.15/<0.10
HT-5	4	1.70/12.00	1.83/10.00	2.02/4.00	2.18/<0.10

* Initial pH of the medium was 2.2.

Table 4. Growth of *Sulfobacillus* sp. strains under autotrophic and heterotrophic conditions

Strain	Number of transfers	
	Growth in the medium with iron without YE	Growth in the medium with YE without iron
HT-1	4	4
HT-3	0	8
HT-4	1	3
HT-5	10	8

genes of the strains under study to the sequences of the known *Sulfobacillus* species (Fig. 5) revealed high homology with the type strain of *S. thermosulfidooxidans* (99.9%). The similarity to other *Sulfobacillus* species was lower (90–98.5%). The strains were therefore classified as members of the species *S. thermosulfidooxidans*.

Structure of the chromosomal DNA. Pulse-field gel electrophoresis profiles of the chromosomal DNA of strains HT-1, HT-3, and HT-5 cleaved with the *Not*1 restriction endonuclease are presented on Fig. 6. All the strains isolated in the present work have unique structures of chromosomal DNA, which differ from those of inoculated *Sulfobacillus* strains [3, 19, 20, 25]. This finding indicates that the inoculated strains were replaced by others that were present in the concentrate. The known restriction profiles for the new strains may be used for their monitoring in biotechnological oxidation of sulfide minerals.

While associations of acidophilic chemolithotrophic microorganisms formed during tank bacterial–chemical oxidation of sulfide ore concentrates have relatively poor species composition, they exhibit significant strain polymorphism, resulting from the microevolution processes under variable environmental conditions.

Years of monitoring of microbial associations in the pulp of the Olympiadinskoe GRF reactors [19, 26] revealed that fluctuations in the content of the major sulfide elements (iron, sulfur, arsenic, antimony, etc.) in the concentrate resulted in changes of the dominant strains. Species composition also changed; for example, different *Sulfobacillus* species have been isolated from the GRF reactors.

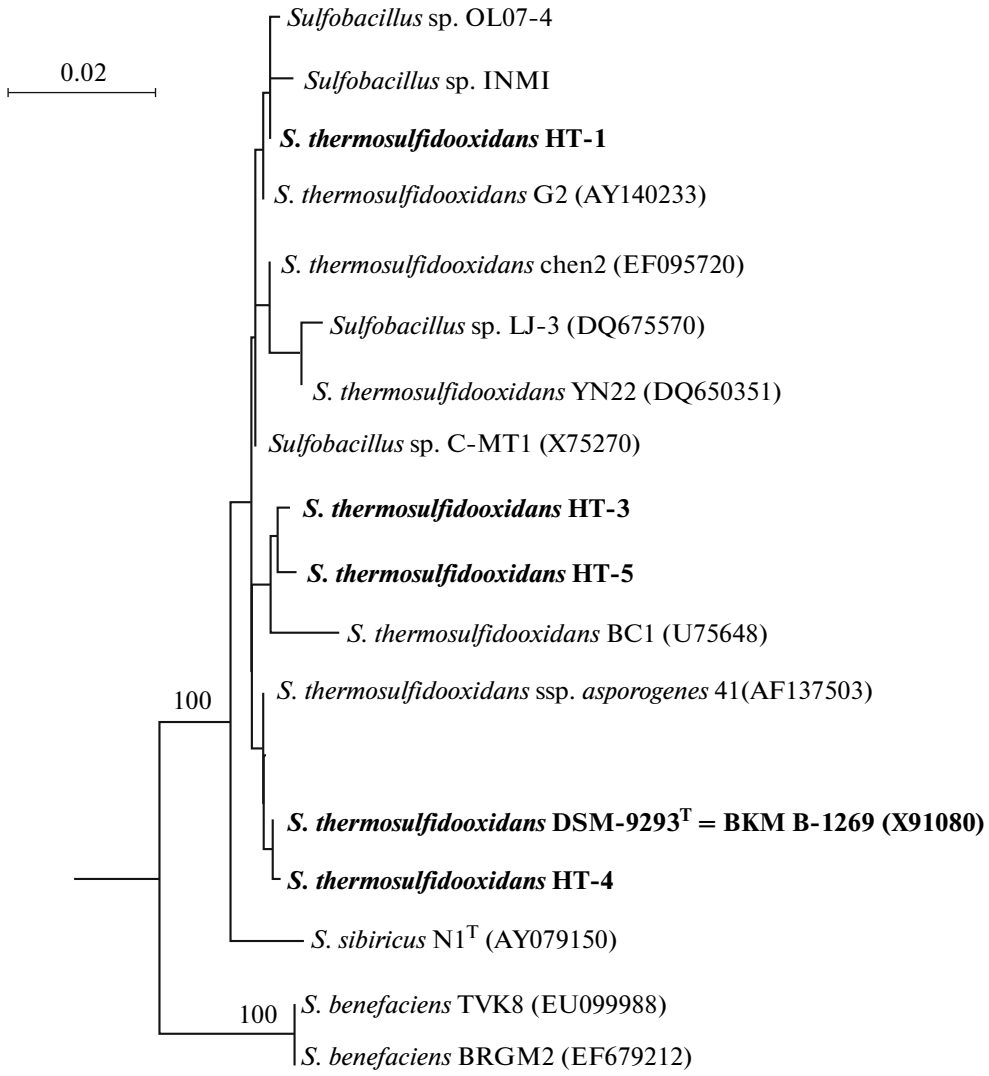

Fig. 5. Phylogenetic position of *S. thermosulfidooxidans* strains (marked by boldface) constructed on the basis of the 16S rRNA gene sequences. The scale bar corresponds to two replacements per 100 nucleotides. The numerals show the significance of the branching order as determined by bootstrap analysis (only bootstrap values above 75 were considered as significant).

The present work demonstrated that variations in the technological conditions of the oxidation of gold–arsenic concentrates result in a new structure of the association, with the known *Sulfobacillus* strains replaced by new strains of *S. thermosulfidooxidans*. This is an indication of significant strain polymorphism within the species, which results in predominance of the most adapted strains during bioleaching of sulfide gold-containing concentrates under different technological modes. The physiological characteristics of the strains suggest that the species is adapted to a wide range of conditions (temperature, pH, and the main energy substrate). Some common characteristics of the strains indicate the possible approaches to increased efficiency of the oxidation processes. For example, an organic carbon source is required for stable growth of all the isolates. The low rates of oxidation of elemental sulfur are noteworthy. Incomplete oxidation of sulfur in the sulfide ore results in increased costs of recovery of noble metals and in an undesirable increase in pH. At pH above 2, growth of the studied strains was suppressed and ferric iron salts precipitated. Introduction of obligately autotrophic microorganisms (*Acidithiobacillus caldus* or *Leptospirillum ferriphilum*), which oxidize sulfur and iron at elevated temperatures and provide organic compounds for the mixotrophic microorganisms, to associations of moderately thermophilic acidophilic chemolithotrophic microorganisms will increase the efficiency of the oxidation processes [27].

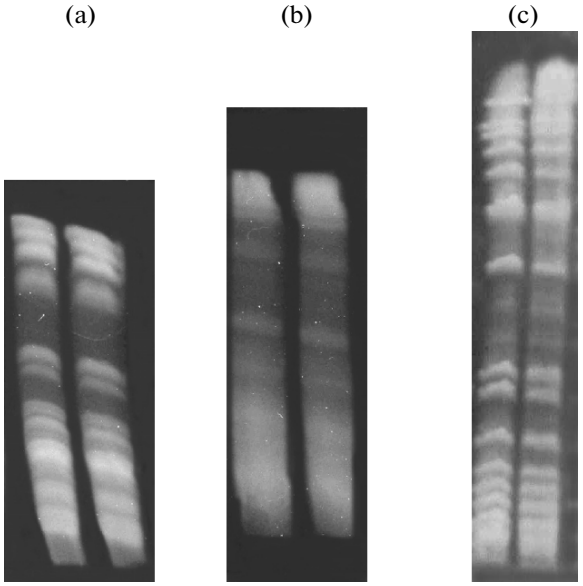

Fig. 6. Pulse-field gel electrophoregram of the fragments of chromosomal DNA of *S. thermosulfidooxidans* strains cleaved with *Not*1 restriction endonuclease: HT-1 (a), HT-3 (b), and HT-5 (c).

Thus, the physiological polymorphism of *S. thermosulfidooxidans* strains indicates the high biotechnological potential of this species for biohydrometallurgy.

ACKNOWLEDGMENTS

The authors thank T.V. Kolganova for 16S rRNA gene sequencing. This work was supported by the Russian Foundation for Basic Research, project no. 10-04-00589.

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