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EXPERIMENTAL ARTICLES

Specific Characteristics of the Strains Isolated from a Thermoacidophilic Microbial Community Oxidizing Antimony Sulfide Ore

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Abstract—Investigation of the phenotypic properties of three mixotrophic bacteria, strains Sb-K, Sb-F, and Sb-S, isolated from an aboriginal thermoacidophilic microbial community participating in biooxidation of ore with high antimony content (26%) and ore concentrates from the Olympiadinskoe deposit under semicontinuous cultivation conditions at 46 ± 1°C, revealed the differentiating characteristics of these strains. The isolated cultures grew lithotrophically through different numbers of transfers: strains Sb-F and Sb-K grew through seven and eight transfers, respectively, and strain Sb-S grew through two or three transfers. Strains Sb-K and Sb-S utilized a wide range of organic substrates for active organotrophic growth during nine or ten transfers, while strain Sb-F was less tolerant to organic compounds. Strain Sb-K grew on a medium with the ore and sulfide ore concentrates in the pH range of 1.0–3.0. Growth of strains Sb-F and Sb-S occurred in the pH ranges of 1.0–2.5 and 1.5–5.5 on media with Fe^{2+} and S^0, respectively.. The optimal initial pH values of the media, corresponding to the maximum specific growth rates, were 1.6–1.7, 1.9, and 2.0–3.0 for strains Sb-K, Sb-F, and Sb-S, respectively. All three strains were able to grow within a broad temperature range, 20–65°C, with an optimum at 46°C (Sb-K), 40–46°C (Sb-F), and 48–50°C (Sb-S). According to the results of DNA–DNA hybridization and phylogenetic analysis, as well as their phenotypic characteristics, the isolates can be classified as novel strains of species of the genus *Sulfobacillus*. Strains Sb-K, Sb-F, and Sb-S, isolated as predominant cultures on the media with sulfide compounds, iron, or sulfur, respectively, were affiliated to the species *S. thermotolerans, S. sibiricus*, and *S. thermosulfidooxidans*.

Keywords: strains of thermoacidophilic bacteria, phenotypic properties, genotypic characteristics.

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The taxonomic composition of highly specialized groups of bacteria participating in iron oxidation and reduction, as well as in the oxidation of reduced inorganic sulfur compounds (RISCs), including sulfide minerals, within a temperature range of 40–60°C is quite uniform. These include bacteria belonging to the genus *Sulfobacillus*, as well as species of three other genera—*Acidithiobacillus caldus* (RISC oxidizer) [2], *Acidimicrobium ferrooxidans* (Fe^{2+} oxidizer) [3], and *Leptospirillum ferrifilum* (Fe^{2+} oxidizer isolated from a 45°C microbial community) [4]. The species composition of the functionally diverse chemolithotrophic thermoacidophilic bacteria of the genus *Sulfobacillus*, class *Bacilli*, order *Bacillales*, phylum *Firmicutes* [5], is represented by a number of species (including five validated species). In addition to the oxidization of the above-mentioned substrates, sulfobacilli are able to oxidize organic compounds, using both oxygen and Fe^{3+} as electron acceptors. In the latter case, RISCs may also serve as electron donors [6, 7]. Moderately thermophilic species of the genus *Sulfobacillus*, *S. thermosulfidooxidans* [8], *S. acidophilus* [9], and *S. sibiricus* [10], as well as the nonvalidated species *"S. thermosulfidooxidans"* subsp. *"asporogenes"* [11], and *"S. yellowstonensis"* [12] and the thermotolerant and mesophilic species *S. thermotolerans* [13], *S. benefaciens* [14], *"S. olympiadicus"* [12], and *"S. montserratensis"* [15], are known. Some of these species comprise more than ten strains. Sulfobacilli were isolated from natural and anthropogenic microbial communities inhabiting sulfide, pyrite–arsenopyrite, pyrrhotite-containing, and polymetallic ores, coal heaps, bioreactors, streams flowing from under the heaps containing polymetallic sulfide ores, etc. However, any literary data on thermoacidophilic chemolithotrophic bacteria participating in the oxidation of ores with high antimony content are unknown. Only the mesophilic chemolithotroph *Acidithiobacillus ferrooxidans* is capable of oxidizing Sb_2S_3 [16]. Like pyrite, antimony ores are considered to be extremely difficult to oxidize. In our studies, when oxidizing gold-containing ores with high antimony content and concentrates from the Olympiadinskoe deposit, we used reactors with mechanical stirring, high-density

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pulp (16%), 45–47°C, and the batch and semicontinuous modes. Enrichment cultures of the microorganisms isolated from the above-mentioned ore (Sb 26%), both during the progressive increase in the concentration of the added substrate for oxidation and adaptation to this substrate, as well as using simultaneous selection of bacteria present in this ore, which preferentially oxidized iron or sulfur, were used as inoculum. With three-stage semicontinuous processing of sulfide minerals of antimony-containing ore and ore concentrates completed, three cultures possessing a certain substrate specificity were isolated. The obtained isolates Sb-K, Sb-F, and Sb-S showed high rates of bioleaching and oxidation of sulfide minerals, iron, or sulfur and were dominant in reactors I, II, and III, respectively. The oxidation degree of sulfide antimony, arsenic, and iron, as well as the level of gold recovery, were high [1]. The goal of the present work was to study the genotypic and phenotypic properties of the three novel cultures of thermoacidophilic bacteria isolated in the course of three-stage semicontinuous cultivation on the medium with antimonite-containing ore and ore concentrates from the Olympiadinskoe deposit.

MATERIALS AND METHODS

Objects of study. The current study was carried out with thermoacidophilic strains Sb-K, Sb-F, and Sb-S isolated in the form of monocultures from a microbial community participating in the semi-continuous process of opening and oxidation of a mixture of the ore (26% Sb) and ore concentrates with high antimony content from the Olympiadinskoe deposit at a pulp density of 16% and 46 ± 1°C. The culture purity was confirmed by microbiological tests and by analysis of the individual restriction profiles of the extracted DNAs. Strains Sb-K, Sb-F, and Sb-S predominated in reactors I, II, and III, respectively, as well as on their respective media (with initial sulfide minerals, ferrous iron, or elemental sulfur). The morphological and some physiological characteristics of the studied strains were similar to those of sulfobacilli [1].

Cultivation. The isolates were grown on a 9K mineral medium [17] containing 0.02% of yeast extract and a relevant optimal oxidation substrate for each isolate. The composition of the mixture of sulfide minerals was as follows: Au, 69 g/t; Sb_S, 10.8%; Fe_S, 7.80%; As_S, 4.76%; and Ca, 5.59%. In the case of strain Sb-K, it was introduced (5%) into conical flasks with the medium (pH 1.7). In the case of strain Sb-K, the pH of the medium with the mineral mixture was preliminary stabilized to the required level with 10 N H_2SO_4, as described [1]. In the second medium (pH 1.8), in which strain Sb-F grew, ferrous iron in the form of $FeSO_4 \cdot 7H_2O$ (9.8 g/l) served as the main energy source. Elemental sulfur (5%) was added to the third medium (pH 2.2–2.35) for strain Sb-S. The bacteria were cultivated in 250-ml flasks containing 100 ml of medium. The inoculum content was 10% (vol/vol). The flasks were incubated on a shaker (180 rpm) at 46°C. Depending on the purpose of the experiments, the strains were grown on the same media without yeast extract (when autotrophic conditions were required) or with a specific organic compound (0.02%) in order to define organotrophic growth. Sulfide minerals, ferrous iron, or sulfur were used for studying lithotrophic growth; sugars, amino acids, organic acids, and complex compounds (peptone, tryptone, casamino acids (amino acids of casein hydrolysate), as well as a number of other compounds, were used for studying bacterial growth under heterotrophic conditions.

Determination of the physicochemical growth parameters. To determine the ranges and optima of pH and temperature of the isolates, 9K medium was used, supplemented with yeast extract and the oxidation substrates, which were used for the isolation of monocultures. The maximal specific growth rates (μ_{max}) corresponded to the optimal pH and temperature values. To study the effect of acidity of the medium, bacteria were grown at 46°C; to determine the temperature limits for growth, strains Sb-K, Sb-F, and Sb-S were grown at pH values of 1.7, 1.8, and 2.3, respectively.

Methods of investigation. During the course of growth, the pH and Eh values were measured with a pH-150M pH-meter (Belarus). The concentrations of ferric and ferrous iron were determined by trilonometric titration [18]; the content of sulfate ion was determined as described in [19]. Bacterial cells were enumerated by the direct count method; their physiological state was examined under a Lumam-I1 light microscope (LOMO, Russia) equipped with a phase-contrast device.

Genotypic characterization of the isolates was conducted as described [7]. To determine the nucleotide composition, the DNA samples of the new strains were extracted and purified according to the standard Marmur procedure [20]. Determination of the nucleotide composition (performed in triplicate) was carried out by the thermal denaturation method (determination of the melting point); the accuracy of the method is ± 0.2–0.5%. The level of DNA–DNA hybridization of bacteria was determined by the standard method of optical reassociation following De Ley [21], which makes it possible to determine the levels of similarity and phylogenetic homogeneity among strains within a species or species independence. The analyses were performed in at least three replicates. The spectroscopic parameters were determined on a Pye-Unicam-1500 spectrophotometer (United States). DNA samples of the novel strains and the type strains of *Sulfobacillus thermotolerans* Kr1^T (VKM B-2339 = DSM 17362), *S. thermosulfidooxidans* 1269^T (VKM B-1269 = DSM 9293), and *S. sibiricus* N1^T (VKM B-2280 = DSM 17363), obtained from the cul-

ture collection of the Laboratory of Chemolithotrophic Microorganisms of the Winogradsky Institute of Microbiology, Russian Academy of Sciences, as well as from other collections, were used.

Phylogenetic analysis. Identification of the three strains, members of the aboriginal microbial association participating in the oxidation of sulfide minerals of antimony-containing ore and ore concentrates (including antimonite) at 46°C, was performed by the analysis of nucleotide sequence of 16S rRNA genes. Amplification and sequencing of the 16S rRNA gene of the isolate was performed using the method described in [22]. Preliminary screening in the GenBank database was performed using the NCBI BLAST software package (http://www.ncbi.nlm.nih.gov/blast). The obtained nucleotide sequences of the 16S rRNA gene fragments were aligned with the sequences retrieved from the GenBank database using the CLUSTALW software package. To compare the de novo obtained sequences with the sequences within the GenBank database, the results of sequencing of the nearly complete 16S rRNA gene sequences of the novel strains were used (approximately 1410 nucleotides, corresponding to positions 15-1435 in *Escherichia coli*). Unrooted phylogenetic trees of the studied bacteria were designed by the methods implemented in the TREECON software package [23].

RESULTS AND DISCUSSION

Earlier, we demonstrated that the exponential-phase cultures of the isolated strains growing under optimal mixotrophic conditions were represented by rod-shaped cells differing in length and diameter, single or arranged in short or longer chains [1]. The vegetative cells of strain Sb-S were the smallest ones (0.4–0.6 × 1.0–1.4 μm), whereas the cells of strain Sb-F were the largest ones (0.8–1.0 × 1.5–2.0 μm). In stationary-phase cultures growing on a mixture of antimonite and sulfide minerals from ore concentrates, the cells of all isolates could be elongated (to 2.0–2.5, 3.0 μm) or contracted to close-to-coccoid cells. The spores were arranged in nonuniformly swollen sporangia. The mature spores of strains Sb-F were oval, the spores of strain Sb-S were oval and spherical, and the spores of Sb-K were ellipsoid and spherical. The latter strain was characterized by rapid sporulation.

Optimal mixotrophic growth of strains Sb-S, Sb-F, and Sb-K occurred during unlimited number of transfers, which is typical of all the previously described sulfobacilli. The specific features of the lithotrophic and organotrophic growth of the isolated cultures are described below.

Lithotrophic Growth and Oxidative Activity

The strains differed in their capacity to grow under autotrophic conditions. Strains Sb-F (which, compared to other isolates, preferably used ferrous iron as an oxidation substrate) and Sb-K (isolated on the medium with sulfide minerals) showed the best lithotrophic growth. Strain Sb-S, which was primarily a sulfur-oxidizing microorganism, survived no more than two to three transfers under autotrophic conditions on the medium with elemental sulfur.

Strain Sb-F grew for seven successive transfers on a medium with ferrous iron as a sole energy source and electron donor. During the first three transfers, the peak rate of iron oxidation was up to 0.15–0.18 g/(l h). The number of cells was $6.1–4.0 \times 10^7$/ml. Spore formation, i.e., the process of cell transition to the state of metabolic rest, was detected. Vegetative cells changed their typical morphology slightly [1]: along with cells of regular size (0.8–1.0 × 1.5–2.0 μm), longer and thinner cells were detected. After transfer 5, strain Sb-F was not able to oxidize the whole amount of iron introduced into the medium. The concentrations of the iron forms, Fe^{3+} and Fe^{2+}, in the culture liquid were 2.66 and 2.80 g/l, respectively; the Eh value was as high as 636 mV. The cell yield was 3.2×10^6/ml. By the end of transfer 7, even when the amount of ferrous iron added to the medium was four times lower, the oxidative activity decreased drastically; growth ceased with the resumption of subculturing.

Strain Sb-K grew under autotrophic conditions on a complex sulfide-containing substrate (ore + ore concentrates) through eight transfers. However, since the above-mentioned sterile substrates may have contained some organic compounds, it seems likely that unambiguous interpretation of the results obtained is impossible. During the first three to four transfers, the cell yield was $6.6–5.1 \times 10^7$/ml; the cells retained their morphology; however, both coccoid and refractory cells were detected in the population. The peak rate of oxidation of newly leached ferrous iron was 0.11–0.13 g/(l h); the rate of accumulation of sulfate ions (the end product of the oxidation of reduced sulfur) was 0.09 g/(l h). The cell yield gradually decreased during subsequent transfers, and, despite complete oxidation of ferrous iron in the course of transfer 6 (0.7 g/l, Eh = 695 mV, cell number = 4.3×10^6/ml), growth practically ceased after transfer 8.

Hence, strain Sb-F, which was isolated as a microorganism that preferentially oxidizes ferrous iron, as well as strain Sb-K isolated on the medium with the mixture of ore and ore concentrates, when cultivated on a shaker, were capable of prolonged lithotrophic growth observed in bacteria of the genus *Sulfobacillus* only in the presence of saturating concentrations of carbon dioxide in the nutrient medium [3, 12, 24–26]. In the case of strain Sb-S, autotrophic conditions were found to be stress-inducing. The strain survived no more than three transfers, which is typical of all *Sulfobacillus* strains.

Table 1. Organotrophic growth of strain Sb-S isolated from an aboriginal microbial community oxidizing the mixture of sulfide minerals of high-antimony ore and ore concentrates at 46°C and pulp density S : L = 1 : 5 under the semicontinuous flow mode

Organic compound	Number of cells per 1 ml ($\times 10^7$)/±, +, −								
	transfer 1	transfer 2	transfer 3	transfer 4	transfer 5	transfer 6	transfer 7	transfer 8	transfer 9
YE	39.1	43.2	38.4	36.4	37.3	35.4	+	+	+
Casam. acids	18.1	29.0	6.1	6.2	6.2	5.9	+	+	+
Tryptone	13.0	32.0	11.0	9.1	9.6	9.7	+	+	+
Peptone	33.0	31.1	7.1	6.8	6.7	+	+	+	+
Sucrose	5.8	22.1	26.2	28.2	25.8	24.1	+	+	+
Glucose	20.1	13.2	13.0	11.1	9.7	+	+	+	+
Ribose	8.0	11.2	10.3	9.9	9.6	+	+	+	+
Glycerol	13.3	25.2	24.4	24.5	22.1	+	+	+	+
Glutathione	4.9	2.6	1.2	1.3	1.2	±	±	−	−
Glycine	6.7	5.5	3.1	3.0	2.9	+	±	−	−
Cysteine	5.2	3.3	1.8 (±)	1.5 (±)	1.1 (−)	−	−	−	−
β-alanine	1.8 (±)	1.2 (±)	1.1 (−)	1.0 (−)	−	−	−	−	−
Glu	1.6 (±)	1.2 (±)	1.0 (−)	−	−	−	−	−	−
Methionine	5.8	4.5	2.2	2.1	1.8 (±)	1.0 (−)	−	−	−
Succinate	2.1	1.2 (±)	1.0 (−)	1.0 (−)	1.1 (−)	1.1 (−)	−	−	−
Malate	1.8 (±)	1.2 (±)	1.1 (−)	1.0 (−)	1.1 (−)	−	−	−	−

Note: YE, yeast extract; Casam. acids, casamino acids, Glu, glutamate. "±", weak (maintenance-type) growth ($1.2–1.9 \times 10^7$ cells/ml); "+," active growth ($>2.0 \times 10^7$ cells/ml); "−", growth was not detected ($\leq 1.1 \times 10^7$ cells/ml).

Organotrophic Growth

The ability of strains Sb-S, Sb-F, and Sb-K to switch from the optimal mixotrophic metabolism to an organoheterotrophic one was studied. For this purpose, the cultures of each strain were grown on 9K medium under optimal mixotrophic conditions, were transferred to media with organic substrates (0.02%) as the sole energy sources and electron donors, and cultivated for nine to ten transfers. The inoculum amount (the number of bacterial cells from the early stationary phase) was 1.0×10^7/ml.

Complex compounds (yeast extract, casamino acids, peptone, and tryptone), carbohydrates (glucose, sucrose, and ribose), individual amino acids (methionine, glutamate, cysteine, β-alanine, and glycine), and organic acids (succinate and malate) were used as the substrates. The effect of glutathione and glycerol on bacterial growth was also studied.

It was found that strains Sb-S and Sb-K were able to switch rapidly from mixotrophic to organoheterotrophic metabolism; however, the number of transfers, as well as the rate of accumulation of the maximum amount of cells, was different (Tables 1 and 2). Strain Sb-F showed the weakest growth under heterotrophic conditions (Table 3).

The most active growth of strain Sb-S, isolated as the predominant culture in reactor III on the medium with elemental sulfur [1], was detected on the medium with organic compounds, including the complex ones (yeast extract, peptone, tryptone, and casamino acids; ten transfers) (Table 1). This may possibly result from the presence of reduced sulfur compounds in these complex substrates. The highest cell yield ($4.3–2.9 \times 10^8$/ml) was detected on the above-listed substrates. The maximum cell yield was detected on yeast extract during transfer 2; growth was maintained for ten transfers, which suggests that yeast extract was the most suitable substrate for organotrophic growth of the sulfur-oxidizing strain. The strain grown on complex substrates retained its morphology typical of the cell populations grown under optimal mixotrophic conditions and was represented by rods ($0.4–0.6 \times 1.0–1.4$ μm) and spores. The amount of spores was 5–25%.

On sucrose, glycerol, glucose, and ribose, the biomass yield was lower, $2.8–1.1 \times 10^8$ cells/ml. The cell morphology was typical of the strain; the amount of prospores and spores increased in subsequent transfers from 2 to 20–35%. Amino acids, depending on the substrates utilized, supported growth of strain Sb-S for two to seven transfers. The best growth was detected on the media with glycine, methionine, cysteine, and glutathione (6.7, 5.8, 5.2, and 4.9×10^7 cells/ml, respectively). Growth of strain Sb-S on other compounds (succinate, malate, β-alanine, and glutamate) was poor or maintenance-type.

The best growth of the second culture, strain Sb-K, which was maintained for nine transfers, was observed

Table 2. Organotrophic growth of strain Sb-K, isolated on the medium with the sulfide minerals of the high-antimony ore and ore concentrates from an aboriginal microbial community oxidizing the above mixture at 46°C and pulp density S : L = 1 : 5 under the semicontinuous flow mode

Organic compound	Number of cells per 1 ml ($\times 10^7$)/±, +, –								
	transfer 1	transfer 2	transfer 3	transfer 4	transfer 5	transfer 6	transfer 7	transfer 8	transfer 9
YE	19.2	18.9	14.9	12.1	11.3	+	+	+	+
Casam. acids	32.4	43.4	45.1	42.7	36.1	+	+	+	+
Tryptone	22.4	7.3	6.3	6.8	+	+	+	+	±
Peptone	4.9	5.1	4.7	4.8	3.2	+	+	±	±
Sucrose	48.8	36.1	12.9	10.0	12.5	+	+	+	+
Glucose	34.6	10.0	12.1	9.4	+	+	+	+	+
Ribose	1.6 (±)	1.1 (–)	–	–	–	–	–	–	–
Glycerol	2.1	2.1	2.0 (±)	1.6 (±)	±	–	–	–	–
Glutathione	1.2 (±)	1.2 (±)	±	–	–	–	–	–	–
Glycine	4.9	4.2	3.2	+	±	±	–	–	–
Cysteine	2.5	2.1	1.7 (±)	1.0 (–)	–	–	–	–	–
β-alanine	1.6 (±)	1.2 (±)	1.0 (–)	–	–	–	–	–	–
Glu	2.1	±	±	–	–	–	–	–	–
Methionine	1.1 (–)	–	–	–	–	–	–	–	–
Succinate	1.8 (±)	1.1 (–)	–	–	–	–	–	–	–
Malate	2.0 ±	1.9 ±	±	–	–	–	–	–	–

Note: See Table 1 for designations.

on the medium with casamino acids (maximum yield of 4.5×10^8 cells/ml; third transfer), sucrose, glucose (4.9 and 3.5×10^8 cells/ml, respectively), and on media with tryptone and yeast extract (2.2 and 1.9×10^8 cells/ml, respectively) in the first transfer (Table 2). The cells growing on the medium with casamino acids had the typical morphology and divided actively; spores and prospores were scarce; and a small amount of swollen, short, refractory cells, as well as ~5% of spores, were detected on the tryptone-containing medium. On media with sucrose and glucose, the number of spores and prospores did not exceed 2–5% of the total bacterial counts. Growth was much weaker (5.1×10^7 cells/ml, transfer 2) on peptone for seven transfers; during the eighth and ninth transfers, only maintenance-type growth was detected. Within five to six transfers, strain Sb-K showed weak growth on media with glycine and glycerol; the rate of oxidation of other substrates was lower.

Comparative analysis of the results obtained showed that the biomass yield of strain Sb-K was much higher only on some substrates (casamino acids, sucrose, and glucose) than that of strain Sb-S in the first transfer; however, the amount of substrates utilized by strain Sb-K for organotrophic growth was lower. Growth similar to that of the two novel strains was previously reported only for one bacteria of the genus *Sulfobacillus*, *S. thermosulfidooxidans* 1269^T [27]; however, the number of transfers and substrates utilized, as well as the cell yield, were much lower.

Strain Sb-F isolated on the Fe^{2+}-containing medium was also capable of switching from the optimal type of metabolism to nonoptimal organoheterotrophic growth; however, unlike the two above-mentioned strains, it showed weaker growth under heterotrophic conditions (Table 3). The most active growth (9 transfers) and the highest cell yield (3.5×10^8/ml) were detected during growth on the medium with yeast extract in transfer 4. The cell morphology was found to be typical ($0.8–1.0 \times 1.5–2.0$ μm), and the amount of prospores and spores was as high as 25–30%. On the medium with casamino acids, growth was also detected within nine transfers; the number of cells in the first transfer was lowest, then reached 1.6×10^8/ml, and remained stable during subsequent transfers (transfers 5–9). The cell morphology was typical; spores were scarce. Tryptone supported bacterial growth only in the first transfer (3.0×10^7 cells/ml) and maintenance-type metabolism during the next four transfers. The bacterial population was represented by vegetative and refractory cells. Growth on peptone was very weak; it was detected only in the first transfer (3.04×10^7 cells/ml). The results obtained demonstrate that the range of complex organic compounds that, under heterotrophic conditions, were suitable for growth of strain Sb-F was much smaller than in the case of strains Sb-S and Sb-K. Among the tested sug-

Table 3. Organotrophic growth of *Sulfobacillus* sp. Sb-F, isolated from an aboriginal microbial community oxidizing the high-antimony ore and ore concentrates at 46°C and pulp density S : L = 1 : 5 under the semi-continuous flow regime

Organic compound	Number of cells per 1 ml ($\times 10^7$)/±, +, −								
	transfer 1	transfer 2	transfer 3	transfer 4	transfer 5	transfer 6	transfer 7	transfer 8	transfer 9
YE	13.1	34.6	33.7	35.1	31.1	30.1	+	+	+
Casam. acids	4.6	11.0	15.1	16.1	15.8	+	+	+	+
Tryptone	3.0	1.8 (±)	1.7 (±)	1.4 (±)	±	−	−	−	−
Peptone	1.4 (±)	1.1 (−)	−	−	−	−	−	−	−
Sucrose	2.1	3.1	4.9	4.2	4.5	+	+	±	±
Glucose	2.2	3.0	2.7	2.4	2.7	±	±	±	±
Ribose	1.0 (−)	−	−	−	−	−	−	−	−
Glycerol	2.8	2.4	2.0	1.9	±	−	−	−	−
Glutathione	1.2 (±)	1.2 (±)	−	−	−	−	−	−	−
Glycine	0.7 (−)	−	−	−	−	−	−	−	−
Cysteine	1.5 (±)	1.3 (±)	1.6 (±)	1.1 (−)	−	−	−	−	−
β-alanine	2.4	2.2	1.8 (±)	1.3 (±)	−	−	−	−	−
Glu	1.1 (−)	−	−	−	−	−	−	−	−
Methionine	2.5	2.4	2.2	2.0 (±)	1.7 (±)	−	−	−	−
Succinate	−	−	−	−	−	−	−	−	−
Malate	−	−	−	−	−	−	−	−	−

Note: See Table 1 for designations.

ars, only sucrose and glucose supported growth and viability of strain Sb-F in nine transfers; however, starting with transfers 8 and 6, respectively, the culture switched to the maintenance-type metabolism. The peak values of biomass yield were 4.9 and 3.0 × 10^7 cells/ml in transfers 3 and 2, respectively. Weak growth (2.4–2.8 × 10^7 cells/ml) was observed for four or five transfers in the presence of glycerol, methionine, and β-alanine. Together with typical cells, a part of the bacterial population was represented by refractory cells. For two or three transfers, only maintenance-type growth (1.2–1.6 × 10^7 cells/ml) was detected on the media with glutathione and cysteine. On the media with ribose, glycine, glutamic acid, succinate, and malate, no growth was detected.

Hence, strains Sb-K and Sb-S were found to be more tolerant to organics and possessed an ability to oxidize a wide range of substrates and a capacity for organoheterotrophic metabolism for numerous transfers, whereas strain Sb-F was more similar to the previously described bacteria of the genus *Sulfobacillus*, which do not exhibit these phenotypic properties.

Determination of the Physicochemical Parameters of Bacterial Growth

Effect of acidity of the medium. Bacteria were grown on optimal media (pH 1.0–5.5) at 46°C. Figures 1a–1c show the results obtained. The differences in the pH range and optima were demonstrated.

Strain Sb-F had the most defined peak of pH optimum for growth (1.9) on the Fe^{2+}-containing medium (Fig. 1a). The μ_{max} value was 0.35–0.36 h^{-1}; the Fe^{3+} concentration in the medium and the Eh value reached their maxima, 4.06 g/l and 867 mV, respectively. The culture grew in a pH range of 1.0–2.3 at a specific rate of 0.08 and 0.26 h^{-1}, respectively; Fe^{2+} was oxidized almost completely, 3.7 and 4.0 g/l; a high redox potential (799 and 859 mV) was detected. At low pH, the cells rapidly became smaller, formed chains and filaments; at pH values higher than the optimal pH, prospores and swollen cells were detected, together with chains of eight to ten cells.

Strain Sb-S grew on the sulfur-containing medium at the initial pH values ranging from 1.5 to 5.0 with a wide range of pH optima (2.0–3.0) (Fig. 1b). At pH levels of 1.5 and 4.0–5.0, the majority of the cells in the growing culture remained viable. At the pH level of 5.0, the medium became considerably acidified (by 3.4 pH units), indicating active oxidation of sulfur under nonoptimal conditions. At optimal acidity, the μ values and the numbers of cells reached their maxima (0.24 h^{-1} and 2.96–3,02 × 10^8 cells/ml, respectively), and the cell morphology was typical.

In the case of long-term cultivation (more than three days) at low and high pH values, nonuniform cell division and the formation of smaller cells, chains, and spores were observed. At pH 5.0, the number of spores was 15% of the total population. At pH 5.5, the culture

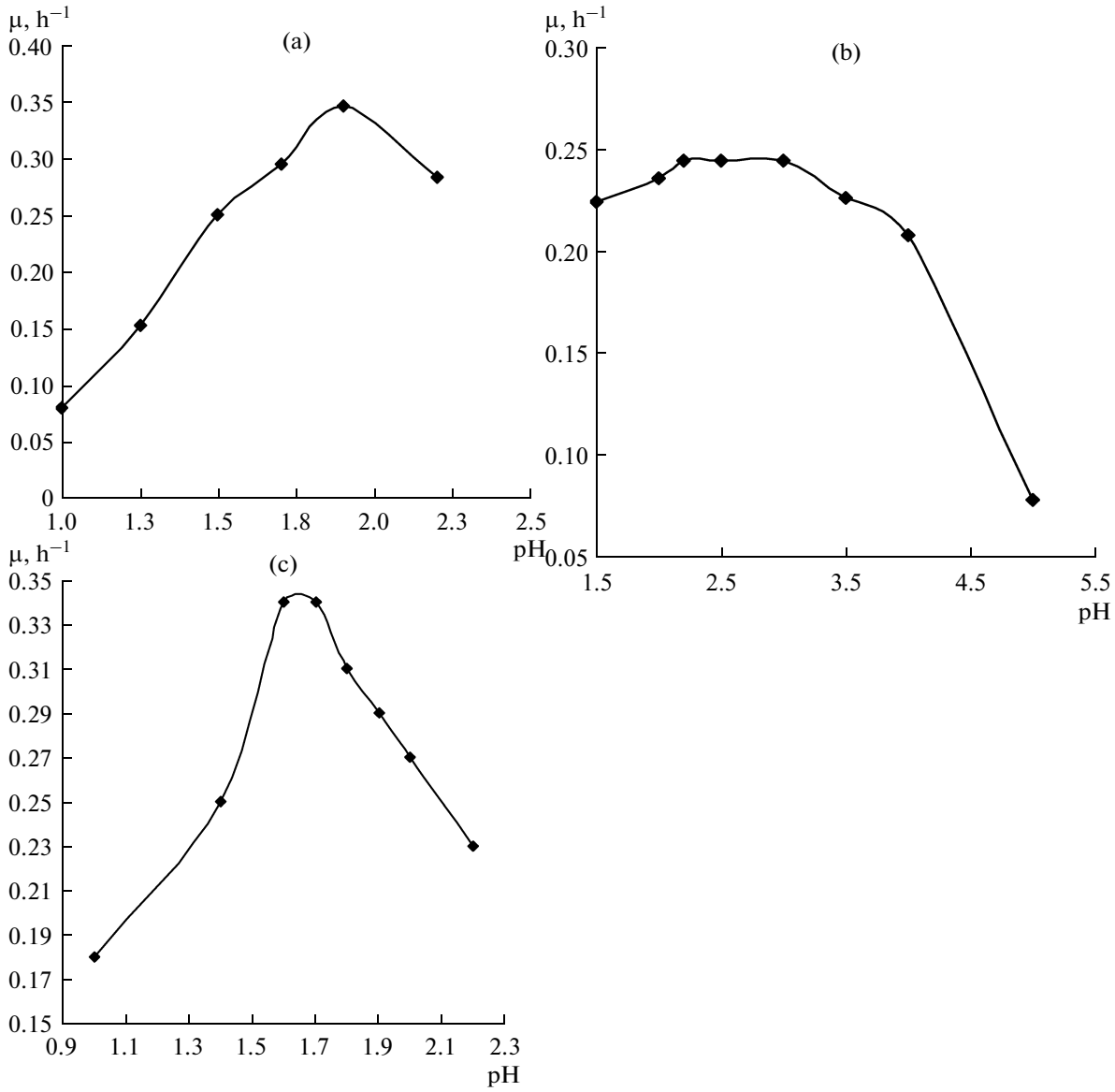

Fig. 1. Correlation between the specific growth rates (μ, h^{-1}) and pH for strains Sb-F (a), Sb-S (b), and Sb-K (c) isolated from an aboriginal microbial community oxidizing sulfide minerals from the mixture of the high-antimony ore and ore concentrates at 46°C and pulp density S : L = 1 : 5 under the semicontinuous flow mode.

remained viable; however, the number of cells decreased by an order of magnitude (as compared to the maximum level) and reached 3.3×10^7/ml.

Strain Sb-K grew on the medium with sulfide minerals (mixture of the high-antimony ore and ore concentrates) in a pH range of 1.0–3.0 and exhibited the highest growth rates (0.33–0.34 h^{-1}) at pH 1.6–1.7 (Fig. 1c). Under these conditions, the Fe^{3+} concentration in the liquid phase was 3.22 g/l, the arsenic concentration was 1.61 g/l, the Eh value was 828 mV. As the initial pH value increased up to 2.3, the growth rate decreased and reached 0.20–0.22 h^{-1}. This strain differed from other isolates in its enhanced resistance to high acidity: it was able to maintain a high growth rate (0.15–0.17 h^{-1}) at pH 1.2. At nonoptimal pH, the cells became smaller; the sporulation level increased as well.

Temperature range and optimum. To determine the temperature growth range and optimum, bacteria were grown at 17–65°C. The diagram (Fig. 2) showing the results obtained demonstrates that the studied cultures differed from each other in these parameters as well.

The growth rate of strain Sb-F on the Fe^{2+}-containing medium at40–46°C was 0.28–0.30 h^{-1} (Fig. 2, curve *1*); the cell yield was $2.56–2.87 \times 10^8$/ml. At 51°C, the μ value was 0.24 h^{-1}; the cell yield was 1.88×10^8/ml. The bacterium grew within a temperature range of 17–62°C. At high temperatures, the cell yield

decreased more rapidly than at low temperatures; however, the decrease in the specific growth rate was more significant at low temperatures (0.004 h^{-1} at 17°C). The culture completely oxidized Fe^{2+} within a wide temperature range (25–53°C). In this case, the Eh value varied within a narrow range (824–842 mV). The highest rate of iron oxidation (0.5–0.6 g/(l h)) was detected at 40–53°C. At low (17–19°C) and high (56–60°C) temperatures, ferrous iron was oxidized incompletely. Within the above-stated range of low temperatures, the population contained mainly small cells and chains, as well as refractory cells and spores. During growth at high temperatures, an increase in the numbers of cells in chains, as well as the formation of long spiral filaments consisting of typical and refractory cells, was detected.

On the S^0-containing medium, the growth rate of strain Sb-S was highest (0.22–0.24 h^{-1}) at 48–50°C (Fig. 2, curve *2*). The highest number of bacterial cells was $3.35–3.59 \times 10^8$/ml. The greatest decrease in pH (from 2.3 to 1.7) was observed. The specific growth rate and the cell yield decreased to 0.14 h^{-1} and 2.35×10^8, respectively, as the temperature increased to 56°C. At 40°C, these parameters were somewhat higher: $\mu = 0.18–0.19$ h^{-1}; the cell yield was 3.04×10^8/ml.

The μ_{max} value of strain Sb-K on the medium with sulfide minerals (46°C) was 0.34 h^{-1}; the cell yield was 3.28×10^8/ml (Fig. 2, curve *3*). Vegetative cells of strain Sb-K retained their typical morphology. The specific growth rate and the cell yield decreased to 0.32–0.28 h^{-1} and $3.12–2.85 \times 10^8$ cells/ml, respectively, as the temperature increased to 51–56°C; that is, the decrease was not as dramatic as in the case of the two other isolates. Within the above-mentioned temperature range, the culture grew at the expense of both iron oxidation (the Fe^{3+} concentration was 2.24 g/l, Eh = 819–828 mV) and the oxidation of sulfur-containing substrates: the decrease in the pH level was significant (0.5 pH units). As the temperature decreased to 40°C, the growth rate and the cell yield decreased to 0.23–0.24 h^{-1} and 2.54×10^8/ml, respectively. At low temperatures, the culture grew due to the oxidation of the iron and sulfur components of the oxidation substrates. Microscopic observations revealed that, in addition to vegetative cells, prospores, spores, and small and refractory cells developed at nonoptimal temperatures.

Summarizing the results of the above experiments, we may conclude that the cultures isolated from the microbial community participating in the semicontinuous oxidation of the sulfide-containing substrate with high antimony content at 46°C differ from each other considerably in their phenotypic characteristics.

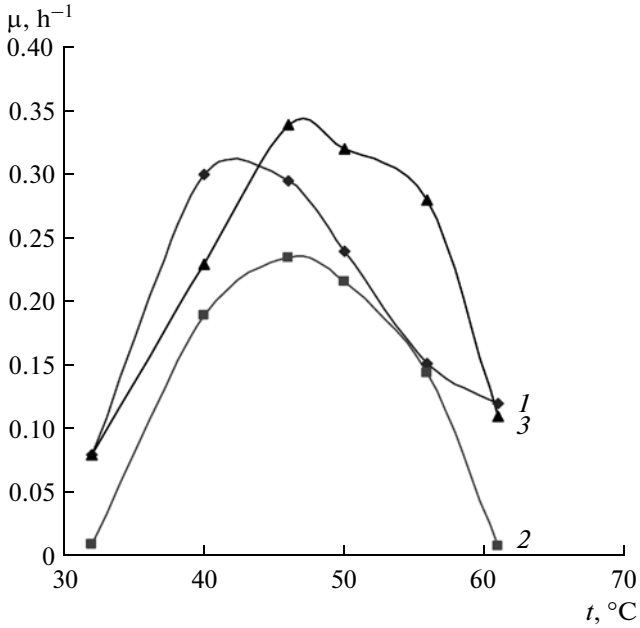

Fig. 2. Correlation between the specific growth rates (μ, h^{-1}) and temperature (t, °C) for three strains, Sb-F (*1*), Sb-S (*2*), and Sb-K (*3*), dominating in each of the three reactors and isolated from an aboriginal microbial community oxidizing the mixture of the high-antimony ore and ore concentrates at 46°C and pulp density S : L = 1 : 5.

Genotypic Characteristics of the Studied Strains

Determination of the DNA nucleotide composition. The results of determination of the DNA nucleotide composition of the novel strains indicate that the DNA G + C contents of these strains differed insignificantly and ranged from 47.6 to 48.1 mol %, as is typical of the *Sulfobacillus* species (Table 4). The average results of the analyses performed in triplicate are presented. The levels of DNA–DNA homology between the isolates were 35–43%, suggesting an interspecific level of relatedness.

DNA–DNA hybridization. To compare the levels of DNA–DNA homology, as well as to confirm that it does not conform to an intraspecific level of relatedness, a series of experiments with type strains of *Sulfobacillus* species was carried out. The results obtained indicated the lack of intraspecific similarity between the genomic DNA of the novel strains and revealed the intraspecific similarity between the studied isolates and the reference strains (Table 4). The level of DNA reassociation between strain Sb-K and the type strain *S. thermotolerans* $Kr1^T$ was 89%. The level of DNA reassociation between strain Sb-F and *S. sibiricus* $N1^T$ was 90% (intraspecific level of relatedness). The level of DNA reassociation between strain Sb-S and *S. thermosulfidooxidans* 1269^T was 85% (while the threshold level of intraspecific similarity is 70–100%) [5].

Table 4. The content of G + C base pairs in the DNA and the level of DNA–DNA homology between the isolates and the reference strains of the type cultures *S. thermotolerans* Kr1^T, *S. thermosulfidooxidans* 1269^T, and *S. sibiricus* N1^T

Strain	DNA G + C content, mol %	Level of DNA–DNA homology, %					
		Kr1^T	1269^T	N1^T	Sb-S	Sb-K	Sb-F
Kr1^T	48.2	100					
1269^T	47.4	33	100				
N1^T	48.2	44	42	100			
Sb-S	48.1	32	85	43	100		
Sb-K	47.6	89	31	39	35	100	
Sb-F	48.0	42	38	90	43	39	100

Analysis of the nucleotide sequence of the 16S rRNA genes. According to phylogenetic analysis of the 16S rRNA gene sequences, all three strains belong to the phylogenetic subdivision of gram-positive bacteria, more specifically, to the phylogenetic cluster containing members of the genus *Sulfobacillus*. The levels of similarity and bootstrap support between the studied strains and some known *Sulfobacillus* species were 98.6–99.9 and 95%, respectively (Fig. 3). The similarity level between the nucleotide sequences of the 16S rRNA gene of strain Sb-K and that of the type strain of *S. thermotolerans* was 99.9%. The level of similarity between the 16S rRNA gene sequence of strain Sb-F and that of *S. sibiricus* was high (98.9%). The similarity level between the 16S rRNA gene sequences of strain Sb-S and the type strain of *S. thermosulfidooxidans* was 98.6%.

Thus, the high level of DNA similarity between the isolated strains and the reference strains, as well as the results of comparative phylogenetic analysis of the 16S rRNA gene sequences, demonstrated the taxonomic affiliation of the isolated bacteria to the known species of the genus *Sulfobacillus*. In Table 5, the properties of the studied and the type strains are summarized.

Hence, significant differences were found between the above-mentioned type strains of *Sulfobacillus* species and the novel isolates in such phenotypic properties as cell size, capacity to grow within a wider temperature and pH range, temperature and pH optima (especially in the case of *S. thermotolerans* and *S. sibiricus*), and the capacity of the novel strains for active litho- and organotrophic growth for nine to ten transfers. Nevertheless, the genotypic properties of the studied strains allow us to assign strains Sb-K, Sb-F,

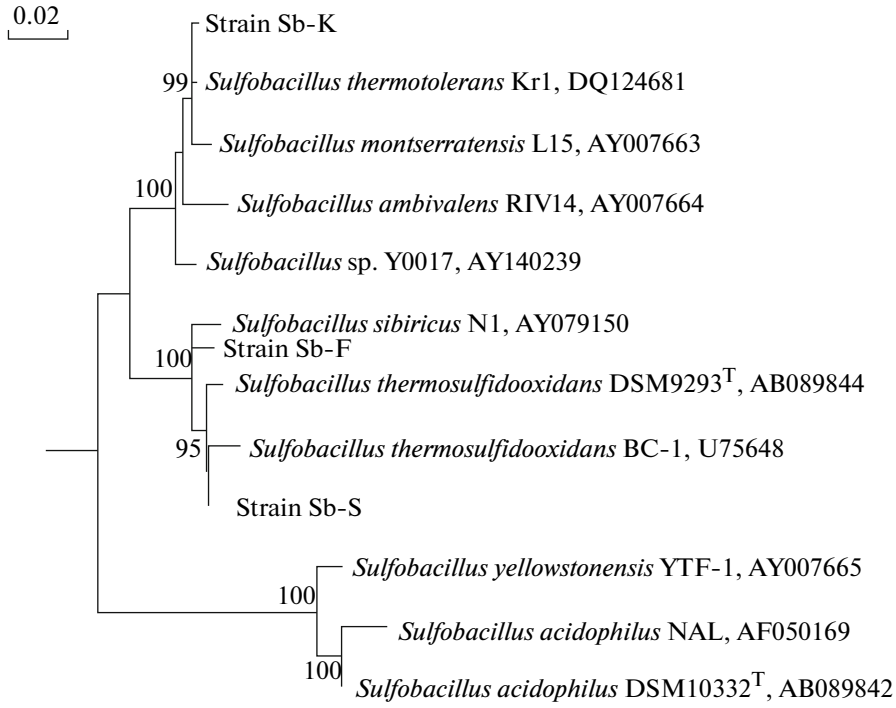

Fig. 3. Phylogenetic position of the strains *Sulfobacillus thermotolerans* Sb-K, *S. sibiricus* Sb-F, and *S. thermosulfidooxidans* Sb-S constructed on the basis of the 16S rRNA gene sequences among representatives of the genus *Sulfobacillus*, cluster *Bacillus-Clostridium* of gram-positive bacteria. The numerals show the significance of the branching order as determined by bootstrap analysis (only bootstrap values above 75 were considered as significant). The bar shows evolutionary distance, corresponding to two substitutions per 100 nucleotides.

Table 5. Differentiating characteristics of strains Sb-S, Sb-K, and Sb-F, isolated from an aboriginal microbial community oxidizing the mixture of high-antimony ore and ore concentrates from the Olympiadinskoe deposit at 46°C and 16% pulp density under the semicontinuous flow mode and those of the type strains of the species *S. thermosulfidooxidans*, *S. thermotolerans*, and *S. sibiricus*

Characteristics	*S. thermosulfidooxidans*		*S. thermotolerans*		*S. sibiricus*	
	Strain Sb-S	Strain 1269^T	Strain Sb-K	Strain Kr1^T	Strain Sb-F	Strain N1^T
Cell size, mm	Rods 0.4–0.6 × 1.0–1.4	Rods 0.6–0.8 × 1.0–3.0	Rods 0.6–0.8 × 1.4–1.8	Rods 0.8–1.2 × 1.5–4.5	Rods 0.8–1.0 × 1.5–2.0	Rods 0.7–1.1 × 1.0–3.0
pH rang (pH optimum)	1.5–5.5 (2.0–3.0)	1.2–5.5 (1.9–2.4)	1.0–3.0 (1.6–1.7)	1.2–2.4 (2.0)	1.0–2.5 (1.9)	1.1–2.6 (2.0)
Temperature range, °C (temperature optimum)	<20–65 (48–50)	<20–58 (50)	17–65 (46)	12–60 (40)	<20–65 (40–46)	20–60 (55)
G + C, mol %	48.1 ± 0.5	47.4 ± 0.15	47.6 ± 0.3	48.2 ± 0.5	48.0 ± 0.4	48.2 ± 0.2
Spore formation	+	+	+	+	+	+
Mineral substrate + yeast extract	Fe^{2+}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, MeS	Fe^{2+}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, MeS	Fe^{2+}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, MeS	Fe^{2+}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, MeS	Fe^{2+}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, MeS	Fe^{2+}, S^0, $S_4O_6^{2-}$, MeS
Organic substrates	YE, peptone, tryptone, casam. acids, sucrose, glycerol, glucose, ribose, glycine, glutathione, methionine, cysteine; (succinate, malate)	YE, casein hydrolysate, glucose, fructose, sucrose, trehalose, mannose, raffinose, glutamate, glutathione	Sucrose, casam. acids, glucose, tryptone, YE, peptone, glycerol, glycine, cysteine, glutamate, malate; (β- alanine)	YE, glucose, fructose, sucrose, glycerol, glutamate, aspartate, cysteine, valine, alanine, glutathione, malate, acetate	YE, sucrose, glucose, casam. acids, tryptone, glycerol, methionine, β- alanine	YE, glucose, fructose, sucrose, sorbitol, glutamate, alanine, glutathione, glycerol
Minimum generation time in the presence of YE, h	2.3 (S^0); 3.7 (Fe^{2+})	6.0 (S^0); 2.5 (Fe^{2+})	3.3 (S^0); 1.8 (Fe^{2+})	1.8 (S^0); 2.0 (Fe^{2+})	3.8 (S^0); 1.8 (Fe^{2+})	3.5 (S^0); 1.4 (Fe^{2+})

Note: Casam. acids stands for casamino acids, YE stands for yeast extract, and MeS stands for sulfide minerals. The substrates that induced weak growth of sulfobacilli are given in parentheses. Organic substrates are arranged in a row by the cell yield in descending order.

and Sb-S to the species *S. thermotolerans, S. sibiricus*, and *S. thermosulfidooxidans*, respectively. The isolated cultures, due to their ability to adapt gradually to antimony, show considerable resistance to Sb, which will be the subject of our further investigations.

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