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EXPERIMENTAL ARTICLES

Functional Diversity of an Aboriginal Microbial Community Oxidizing the Ore with High Antimony Content at 46–47°C

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Abstract—A procedure for rapid (7–10 days) obtaining of enrichment cultures of aboriginal thermoacidophilic microbial communities from ores with high antimony content (Sb 26%) was developed. This technique allows for rapid alkalization of the medium due to the abundance of calcites, as well as the low antioxidant status of the initial cells. The ore concentration in the medium was gradually increased to 10 g/l. In the course of this process, selection of enrichment cultures containing microbial strains preferentially oxidizing ore, S_0, or Fe^{2+} is carried out. A combination of three enrichment cultures allowed us to rapidly (in six days) adapt the aboriginal strains to high-density pulp (16%) in the reactor at 46°C, as well as to carry out a three-stage semi-continuous cultivation in the reactors at $D = 0.0042\ h^{-1}$ and to isolate from each reactor the pure cultures of predominant bacteria involved in the process of bioleaching/oxidation of the mixture of antimonite-containing ores and sulfide flotation concentrates. It was demonstrated that, in the microbial community of reactor I, strain Sb-K exhibiting high rates of growth and initial substrate oxidation was predominant. In reactor II, strain Sb-F prevailed, showing a high substrate specificity with respect to Fe^{2+}. A sulfur-oxidizing strain involved in active oxidation of reduced inorganic sulfur compounds (RISCs) was predominant in reactor III. Nevertheless, together, all three strains showed synergism and were able to oxidize S^0, Fe^{2+}, and sulfide minerals (including antimonite Sb_2S_3 in the presence of 0.02% yeast extract) in reactors. The strains differed from each other in their DNA restriction profiles, growth rates, and the rates of inorganic substrate oxidation under mixotrophic conditions. The phenotypic properties of all the studied isolates have a certain similarity to those of sulfobacilli.

Keywords: antimony sulfide ore, reactors, processing regimes, aboriginal microbial community, isolates, trophic functions.

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Microbial communities of the ecotopes with sulfide gold-containing ores developed in the course of evolution. The successful implementation of a biotechnology for metal recovery depends on the preservation of the physiological potential of the aboriginal bacterial communities. The pathway of sulfide mineral processing, including recovery, leaching, and oxidation, determines the main trophic pathway in the community in which energy sources (Fe^{2+} and reduced sulfur compounds) alternate with each other, or, at some stage of the trophic chain, one of them becomes predominant. The first stages may be implemented in noncontact (with Fe^{3+}), contact (with microorganisms adhered to the surfaces of mineral particles), or combined modes [1], whereas the final degree of sulfide mineral oxidation requires high levels of the enzymatic activity of specific bacteria, members of the aboriginal community. At 45–50°C, bacteria of the genus *Sulfobacillus* prevail among the chemolithotrophs [2–4]. These bacteria belong to a unique and, at the same time, universal group of extreme acidophilic organisms highly resistant to heavy metal ions [2, 5] and capable of oxidizing sulfide minerals Fe^{2+}, reduced inorganic sulfur compounds (RISCs), and organic compounds (at low concentrations) under aerobic conditions within a temperature range of 20–60°C. Under hypoxic and anoxic conditions, they are able to oxidize RISCs and organic compounds and simultaneously reduce Fe^{3+} [4, 6, 7]. Pyrite (FeS_2) and antimonite (Sb_2S_3) are considered to be extremely difficult to oxidize. These minerals, together with pyrrhotite (FeS) and arsenopyrite (FeAsS), are the main constituents of gold-containing sulfide ores from the Olympiadinskoe deposit. Bacterial transformation of antimonite from gold–antimony concentrates to antimonous trioxide results in the formation of a material suitable for cyanidation [8], while incomplete oxidation of antimony-containing sulfide minerals to other oxides and hydroxides of low solubility results in high cyanide consumption and gold losses [9]. Isolation and utilization of thermoacidophilic microorganisms

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with high kinetic characteristics with respect to the oxidant (Fe^{3+}) production and transformation and oxidation of RISCs will permit evaluation of the functioning of members of the aboriginal bacterial community at certain stages of processing.

The aim of the present work was to study biooxidation of ores with high antimony content from the Olympiadinskoe deposit at 46–47°C as well as to describe the trophic diversity of microorganisms from the aboriginal bacterial community involved in this process.

MATERIALS AND METHODS

Obtaining of an enrichment culture. An enrichment culture of aboriginal microorganisms was obtained at 46°C on medium with gold-containing pyrrhotite pyrite–arsenopyrite ore with high antimony content (26%) from the Olympiadinskoe deposit. The liquid phase of the medium was a 9K mineral medium (pH 1.8) [10] supplemented with 0.02% yeast extract. The ore contained the following: Au, 16 g/t; Sb, 26%; Fe_{total}, 1.04%; Fe_S, 0.88%; As_{total}, 0.3%; As_S, 0.09%; S_{total}, 12.58%; S_S, 11.18%; S_0, 1.42%; Ca, 6.67%; and C, 3.99%. The ore contained mainly antimonite and trace quantities of arsenopyrite, pyrite, and pyrrhotite as oxidation substrates. A high calcite concentration that may cause rapid alkalization of the medium is noteworthy. Weighed portions (1–5 g) of ground ore were placed in 250-ml flasks containing 100 ml of the above-mentioned medium without additional inorganic energy sources. At the same time, in order to obtain enrichment cultures from the ore, as well as to simultaneously carry out the selection of the microorganisms exhibiting the highest rate of oxidation of S_0 and Fe^{2+}, weighed portions of ore were placed in flasks with the medium containing sterile elemental sulfur (0.5–1.0 g; pH 2.3) or Fe^{2+} as ferrous sulfate (1–3 g; pH 1.8). All flasks with ore samples were incubated in a thermostat at 46°C. To maintain an optimal pH value (1.8–2.3), as well as to minimize the negative effects of reactive oxygen species (generated under intense aeration at high temperatures) on development of the aboriginal organisms, a new approach was developed. Periodic alternation of the aeration degree was applied as follows: dynamic conditions (incubation on an Inkubator 1000 rotary shaker (Heidolph, Germany) at a rotation speed of 180 rpm) were changed to static conditions and vice versa. Every 0.5–1 h, samples were taken from each flask, pH was measured, and microscopic examinations were carried out. It was established that, after 1-h incubation, the pH level sharply increased up to 6.0 and higher under dynamic conditions; it increased especially quickly (after 0.5-h incubation) during the isolation of enrichment cultures from weighed portions of ground ore exceeding 2 g. Therefore, the cultures were henceforth isolated from 1-g samples, pH was measured every hour, the acidity of the medium was adjusted to the required values by adding 10 N H_2SO_4 to the liquid phase, and varied aeration conditions were used. During the first stages of isolation of the enrichment cultures of aboriginal microorganisms, static conditions were preferential; dynamic conditions were used for a short period of time for pulp agitation, separation of bacterial cells from ore particles, and increasing the oxygen content in the media. As the population grew, the flasks were additionally supplemented with 1-g portions of ore under constant pH control and microscopic examinations. The final concentration of ore in the pulp was up to 10%. Three parallel aboriginal communities were isolated in the presence of ore alone, ore + iron, and ore + sulfur combined, and used as an enrichment culture. Three enrichment communities were maintained separately, in order to preserve the aboriginal microorganisms of each variant selected according to their ability to rapidly oxidize "their own" substrates and to actively grow on it at a pulp solid : liquid (S : L) phase ratio of 1 : 20.

Adaptation of the enrichment culture to high concentrations of sulfide minerals at 46°C. To adapt the enrichment culture to growth at a sulfide mineral concentration of 16%, i.e., to the high density of the pulp with the S : L ratio of 1 : 5, a 3-l reactor containing 1 l of the medium (mineral 9K medium with 0.02% yeast extract, pH 1.8) was used. The batch regime was used. A mixture of ore with high antimony content and two flotation concentrates from the Olympiadinskoe deposit (1 : 1 : 2 wt/wt) was used as an oxidation substrate. The mixture contained the following : Au, 69 g/t; Sb_S, 10.8%; Sb, 12, 8%; Fe_S, 7.80%; As_S, 4.76%; and Ca, 5.59%. The complex inoculum (10% vol/vol) consisting of the three aboriginal communities grown on ore, ore + iron, and ore + sulfur (2 : 1 : 1) was introduced into the reactor; the cell number was 1.5×10^8/ml of the initial medium. The density of the pulp with the initial S : L ratio of 1 : 20 (4.8%) was increased by adding the substrate, taking into account the process parameters. The pH and Eh values were measured; the concentrations of Fe^{3+} and Fe^{2+}, as well as the cell numbers and changes in the physiological state of microorganisms during oxidation, were analyzed. The pulp in the reactor was agitated using a stirrer (rotation speed 450 rpm). The temperature (46–47°C) was controlled using an ultrathermostat. Air was supplied with a compressor at a rate of 3 l/min. Evaporation was minimized using a water cooler. During adaptation of the microbial community to high densities of the substrate for oxidation (in the pulp), the principle of mass exchange was applied: the composition of the liquid phase was altered three times after collecting a third of the pulp volume from the reactor, centrifugation, and returning the solid phase with microorganisms and fresh medium to the reactor. These procedures were aimed at increasing the concentrations of nitrogen, phosphorus, and magnesium in the pulp, as well as at decreasing the concentrations of metabolites and heavy metal ions and as potential toxic agents and

growth inhibitors. After high density was achieved, the bioleaching/biooxidation of sulfide minerals was carried out for two days.

Semicontinuous oxidation of sulfide minerals by the thermoacidophilic microbial community at 46–47°C. At this stage of the investigation, the microbial community actively involved in antimonite oxidation and tested in the course of the batch process in the reactor at a pulp density of 16% was used. The regime simulating the continuous one was used in three successively installed laboratory reactors (the description of the reactors and cultivation parameters see above) with the total pulp volume of 3 l. The first reactor was used as a charging one. As the pulp density was 16% in reactor I (see above), 300 ml of the pulp was transferred from reactor I to reactor II; then, 300 ml of the fresh pulp was fed to reactor I. This procedure was repeated until the pulp densities in reactors I and II were equal. The same procedure was used for pulp density (up to 16%) in reactor III. The initial substrate was introduced into reactor I, and the feed-batch mode of the semicontinuous process in the reactors was used. Each time, 300 ml of the pulp were replaced under constant control of the parameters until the pulp densities became equal in all reactors. The complete pulp replacement in reactor III was achieved within 240 h after the onset of bioleaching and biooxidation (BO) of sulfide minerals. Hence, the flow rate was 0.0042 h^{-1}.

Analytical methods. During the growth of the microbial community, the pH and Eh values were measured using a pH-150M pH-meter (Belarus). The concentrations of ferric and ferrous iron were determined by trilonometric titration [11]. The total arsenic concentration in the liquid phase of the pulp was determined by iodometric titration (with immobilization of iron ions with $TiCl_3$) [12]. The concentrations of sulfate ions and the enzymes of sulfur metabolism were determined as described in [13, 14]. Bacterial cells were enumerated by direct counting; their physiological state was examined under a Lumam-I-1 light microscope (LOMO, Russia) equipped with a phase-contrast device. Electron microscopic observations were carried out as described in [15]. At the end of the process, the material composition of the biocake (solid residue resulting from bacterial oxidation of sulfide minerals) was analyzed at the ZAO Polus laboratory in order to determine the degree of oxidation of sulfide antimony, iron, and arsenic. The degree of gold recovery by cyanidation was determined as well. Sorption cyanidation was carried out at 20°C for 48 h under the following conditions: pulp acidity of 30% (wt/vol), pH 10.2–10.5 (adjusted with NaOH), cyanide (NaCN) concentration of 1 g/l, aeration of 25 l/h, and sorbent (Norit 3515 coal) concentration of 8%.

Isolation of members of the thermophilic microbial community involved in the oxidation of antimony sulfide ore. Functional diversity of the obtained isolates. The pure cultures of bacteria from the aboriginal microbial community in each reactor was obtained by serial 3-fold dilutions in three replicates. We used 9K mineral medium with yeast extract (0.02%) as a growth factor and three different energy substrates, including: (i) the aforementioned antimonite-containing substrate (5 g/l; pH 1.7), (ii) ferrous iron as $FeSO_4 \times 7H_2O$ (9.8 g/l; pH 1.8), and (iii) elemental sulfur (1%; pH 2.3). The monocultures were isolated from three reactors and grown in flasks on a shaker (180 rpm) at 46°C. In addition to the terminal dilution method, pure cultures were obtained by inoculation on the special medium initiating sporulation in acidophilic chemolithotrophic bacteria [16]. This technique allowed us to trace the process of spore formation, as well as to characterize it when describing the morphology of the isolated bacteria. To isolate a culture from a single cell, the spores were boiled at 100°C for 35 min and inoculated into the above-mentioned media at 46°C; three repeats of serial tenfold dilutions were used. The cultures were tested for purity as described in [3].

Functional diversity of the isolates was studied using sulfur- and iron- containing compounds as oxidation substrates. These compounds were added to the liquid 9K medium prior to incubation on a shaker at 46°C. The following mineral substrates were used: elemental sulfur, antimonite, tetrathionate, thiosulfate, and ferrous iron, as well as the initial mixture of ore and ore concentrates used for cultivation in the reactors. On the Fe^{2+}- or S_0-containing media, the cultures were grown under mixotrophic conditions in the presence of yeast extract (0.02%) for 2 days; on the media with the sulfide minerals of antimony ore and flotation concentrates, $S_2O_3^{2-}$ and $S_4O_6^{2-}$, the cultures were grown for 3 days; and on the media with antimonite, the cultures were grown for 14 days. The initial concentration of sulfide minerals of the mixture or antimonite was 10 g/l; the initial concentration of Fe^{2+} and S_0 was 5 g/l; the initial concentration of $S_4O_6^{2-}$ and $S_2O_3^{2-}$ was 10 mM. Using the results obtained, the maximum growth and oxidation rates were calculated as described in [3, 13, 17]. The experiments involving exchange of the sulfate ion (in the initial medium) for the chloride ion were used as the control of the S^{2-}/S^0 oxidation to the terminal products, sulfate ions.

The tables and figures show the results of the typical experiments performed in triplicate.

RESULTS AND DISCUSSION

Isolation of an Enrichment Culture from the Aboriginal Microbial Community Involved in the Oxidation of Sulfide Ore with High Antimony Content

Accurate application of the new method, including gradual increase of the ore content in incubation flasks, maintenance of the aeration regimes, and adjustment of the pH of the media, allowed us to detect vegetative cells after two to three days of incuba-

Table 1. Conditions and parameters of bacterial oxidation of the sulfide minerals of the high-antimony ore and gold-containing flotation concentrates from the Olympiadinskoe deposit by the aboriginal microbial association in the course of its adaptation to the pulp density of 16% in the reactor under the batch regime (46°C)

No.	Process parameters	Units of measure	Parameter values obtained in the course of bacterial oxidation in the reactor
1	Pulp density	S : L	1 : 20 to 1 : 5
2	Reactor volume/Working volume	l/l	3/1
3	Grade and particle size of the concentrate	%, mm	80%, –0.044 mm
4	Agitation level	rpm	450
5	Total amount of microbial cells in the association	cells/ml	1.19×10^8–1.34×10^8
6	Total iron concentration	g/l	7.23
7	Active acidity of the liquid phase of the pulp	pH units	1.80–1.33
8	Redox potential	mV	782–830
9	Total arsenic concentration	g/l	3.06
10	Duration of the adaptation process	days	6

tion at 46°C. After 7–10 days of incubation, dividing cells were predominant in all media; iron recovery and oxidation were detected; and a decrease in the pH level and an increase in the population density were observed. The growth rate of the population increased under intense aeration of the medium on the shaker. The enrichment culture obtained with the ore as an energy substrate was subcultured on fresh medium containing 5% of the ore; the total number of cells in the enrichment culture was 7.44×10^7/ml. Bacterial cell numbers in the cultures grown in the media supplemented with ferrous iron or sulfur were 9.95 and 9.08×10^7/ml, respectively. Henceforth, only the complex inoculum was introduced into the reactors. The inoculum consisted mainly of the microbial population grown on the ore-containing medium and supplemented with the population of selected microorganisms isolated from ore samples and grown on iron- or sulfur-containing media, and involved in active oxidation of these substrates (and, therefore, in the active generation of the oxidizer, Fe^{3+}, and in providing the required acidity). The mixed enrichment culture consisted of rod-shaped bacteria and the cells of *Ferroplasma acidiphilum*-like archaea [18].

Thus, the new technique for the isolation of aboriginal microbial associations from ores with high antimony content, despite various negative effects (alkalization of media, intense aeration) on the initially small population growing at 46°C, allowed us to achieve the functional diversity and selection of isolates at the first stage, as well as to induce rapid development of iron-, sulfur-, and sulfide-oxidizing strains in the community.

Adaptation of the Enrichment Culture of Aboriginal Microorganisms to a Pulp Density of 16% At 46°C

During the adaptation process, the pulp density was increased daily from 4.75% to 14%. At the last stage (from 14 to 16% of pulp density), the process of adaptation took two days. The high pulp density corresponded to the solid : liquid (S : L) phase ratio of 1 : 5 in the cultivation medium. Table 1 shows the adaptation parameters of the aboriginal microbial community determined in the course of pulp density increase. For the enrichment culture, the duration of adaptation to the high-density pulp in the reactors was 6 days. The rapid increase in the sulfide mineral content (16%) was due, apart from the use of the fast-growing enrichment culture, also to mass exchange, gradual addition of solid material, and constant monitoring of the growth parameters. The results obtained demonstrated that, in the course of oxidation of sulfide minerals at increasing pulp density, the number of bacterial cells in the total population was maintained at 1.19–1.34×10^8/ml. The number of archaeal cells (5×10^3/ml) gradually decreased; archaeal growth ceased at the pulp density of 8%. During BO of sulfide minerals, the H^+ concentration increased from 1.8 to 1.33 pH units. The total amount of oxidized iron and arsenic reached 7.23 and 3.06 g/l, respectively. The redox (Eh) value was 782–830 mV, suggesting that iron-containing substrates in the liquid phase were completely oxidized. The mentioned Eh values of the electrolyte (liquid phase of the pulp) promoted oxidation of the sulfide minerals with a high electrochemical potential (pyrite and antimonite).

On completion of the adaptation process, the efficiency of bioleaching/oxidation of sulfide minerals by the microbial association at 46°C was monitored in the reactor operated in batch mode for two days. Table 2 shows the data on the oxidation kinetics of the above-mentioned substrates. It is obvious that the oxidation of sulfide minerals was quite intense. There was a constant decrease in the pH level. For the normal operation of the process, the pH level was increased up to the optimum (pH 1.8) after 24-h cultivation. Within another 24 h, the pH level decreased to 1.42, which

Table 2. Oxidation of the pulp (16%) from the mixture of high-antimony ore and flotation concentrates from the Olympiadinskoe deposit by thermophilic microbial association in the laboratory reactor during batch cultivation at 46°C

Oxidation duration (days)	pH	Eh, mV	Fe^{3+}/Fe^{2+} concentration, g/l	Arsenic concentration, g/l	Total amount of microbial cells in 1 ml, $\times 10^8$
1*	1.58 → 1.8	824	4.34/0	1.11	1.20
2	1.42	830	5.24/0	1.50	1.21

* Mass exchange of 1/2 of the liquid phase of the pulp after 24-h cultivation.

indicates that the active oxidation of sulfur occurred, which resulted in the accumulation of sulfate ion (the terminal product of sulfur oxidation) in the liquid phase. The high Eh value (830 mV) reflected the changes occurring in the pulp and associated with the iron and antimonite oxidation. The concentrations of iron and arsenic in the liquid phase of the pulp increased. Ferrous iron was not detected. The results of microscopic examinations demonstrated that approximately 50% of the cell population consisted of refractory resting cells. The physiological state of microorganisms was improved due to mass exchange of at least half of the liquid phase of the pulp. However, within another 24 h, despite the fact that the total number of cells in the population was the same, the amount of resting forms increased to 40–45% once more; prospore-containing cells, mature spores, and refractory cells were detected. Vegetative cells of 0.8–1.0 ± 1.2–2.0 μm, smaller motile cells, and chains consisting of several cells that had not separated after division, as well as old and lysing cells, were detected. The overall pattern corresponded to that of the bacterial population which we have previously observed during the oxidation of antimony-containing ores with high antimonite content (39%) under batch conditions at high temperatures due to the inhibitory effects of antimony, arsenic, and other ions of heavy metals (occurring independently or in complexes with organic acids) on thiol-containing compounds in bacterial cells. Denaturation resulted from cleaving the intraprotein bonds responsible for the maintenance of the tertiary and secondary structure [19].

Semicontinuous Oxidation of Sulfide Minerals by the Thermoacidophilic Microbial Community in the Three Laboratory Reactors at 46° ± 1°C

To study the trophic spectrum and growth strategy of the microbial community during the oxidation of sulfide minerals with high antimony content from the Olympiadinskoe deposit, semicontinuous cultivation of the thermoacidophilic microbial community was carried out. Figure 1 shows the kinetic parameters of semicontinuous bacterial oxidation of the mixture consisting of sulfide minerals with high antimony content and two flotation concentrates (1 : 1 : 2 wt/wt) from the Olympiadinskoe deposit by the microbial association in the course of three-stage semicontinuous cultivation under feed-batch mode at 46 ± 1°C. The values of the parameters in Fig. 5 correspond to every 24 h of cultivation, although determination of the parameters and pH correction were carried out throughout the whole day.

The total amount of cells at the beginning of the semicontinuous process of bacterial oxidation of sulfide minerals was 1.31×10^8, 1.19×10^8, and 1.04×10^8 cells/ml (in reactors I, II, and III, respectively); i.e., it decreased insignificantly during the processing (Figs. 1a–1c, curves *6*). The cells of moderately thermophilic rod-shaped bacteria (0.4–1 ± 1.0–2.0 μm) were the main constituents of the microbial population. In addition, smaller bacterial cells and archaea were detected (the latter were irregularly present in reactors I and II); their maximum amounts were 1×10^6 and 2×10^3 cells/ml, respectively. At the end of continuous cultivation, the association of thermophilic microorganisms consisted of vegetative cells (70–75%), occurring singly or in chains, as well as of sporulating cells, spores, and refractory forms.

Oxidation of sulfide minerals in the course of three-stage cultivation of the microbial community was characterized by the following parameters of the liquid phase of the pulp. In reactor I, the pH values ranged from 1.60 to 1.95 (1.68 on the average) (Fig. 1a, curve *1*). The pH level was adjusted with 56.0 ml concentrated sulfuric acid before charging. In reactors II and III, acidity increased from one reactor to another and varied from pH 1.63 to 1.43 (1.52 on the average) (Fig. 1b, curve *1*) and from pH 1.52 to 1.30 (1.37 on the average) (Fig. 1c, curve *1*), respectively. Hence, the pH values in the reactors suggest that the processes of sulfide minerals recovery, release of ferrous iron and arsenic to the liquid phase of the pulp, as well as of oxidation of iron, sulfur, and the products of sulfur transformation, were quite intense. During the first two days of cultivation, the rates of microbial oxidation for arsenic and ferrous iron (leached from fresh concentrate) were high. The concentration on arsenic in the liquid phase ranged from 1.27 to 1.87 g/l (Figs. 1a–1c, curve *5*). Complete oxidation of Fe^{2+} to Fe^{3+} (2.66–5.28 g/ml) was observed, as demonstrated by the absence of ferrous iron in the liquid phase of the pulp (Figs. 1a–1c, curves *3* and *4*). The Eh values were high, reaching 758–763 mV in reactors I and II (Figs. 1a and 1b, curve *2*) and 752–773 mV in reactor III (Fig. 1c, curve *2*). During further processing, the

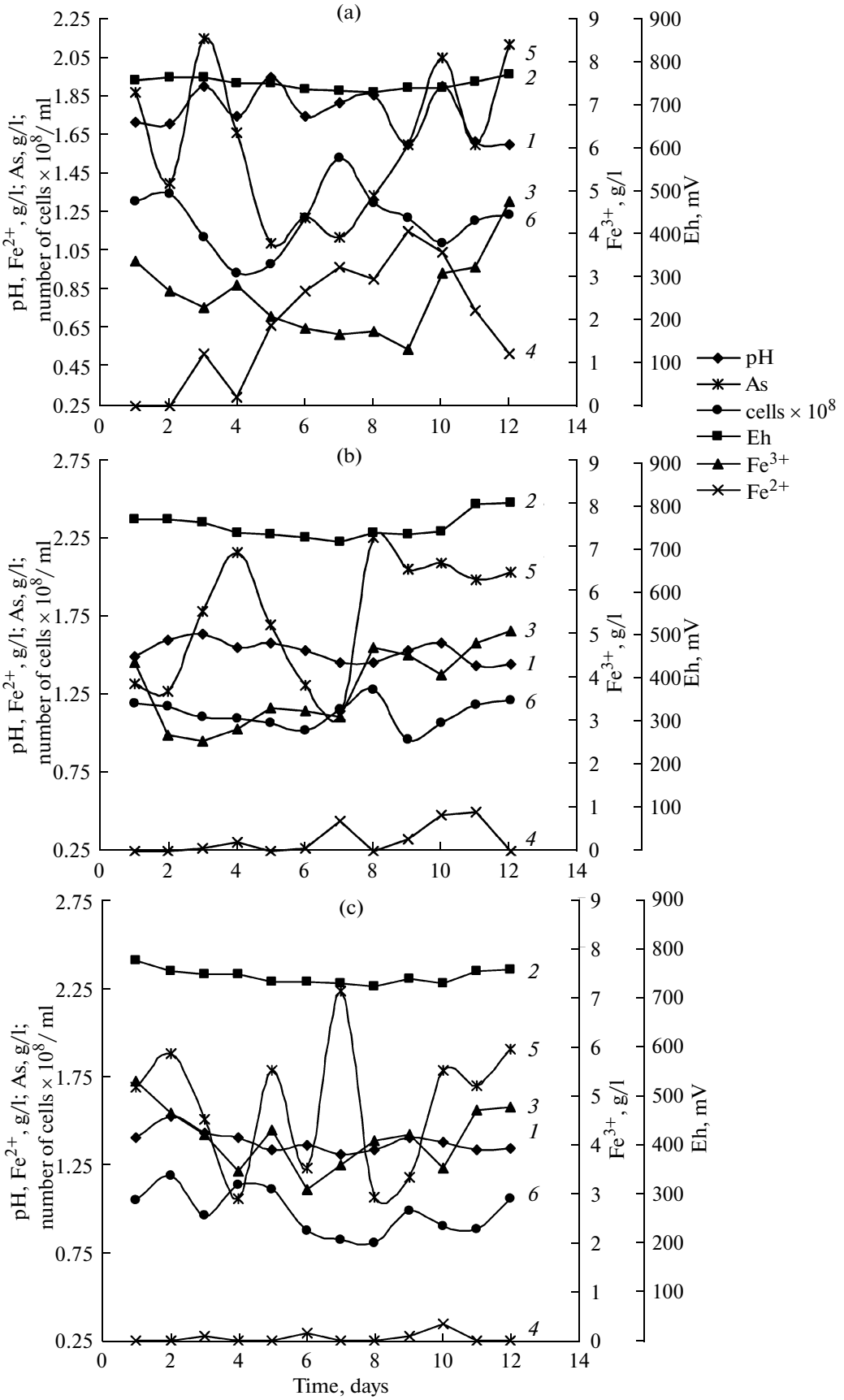
(a)
(b)
(c)
pH, Fe^{2+}, g/l; As, g/l; number of cells × 10^8/ ml
Fe^{3+}, g/l
Eh, mV
Time, days
pH
As
cells × 10^8
Eh
Fe^{3+}
Fe^{2+}
1
2
3
4
5
6

Fig. 1. Kinetic parameters of bacterial oxidation of sulfide minerals from the mixture of high-antimony ore and flotation concentrates from the Olympiadinskoe deposit by the thermoacidophilic microbial association in high-density pulp (16%) at 46°C ± 1°C during three-stage semicontinuous cultivation at D = 0.0042 h^{-1}. Biooxidation in reactors I (a), II (b), and III (c): pH of the pulp (*1*); Eh (*2*); Fe^{3+} concentration, g/l (*3*); Fe^{2+} concentration, g/l (*4*); As concentration, g/l (*5*); number of cells 1 × 10^8/ml (*6*).

presence of both Fe^{3+} and Fe^{2+} in the liquid phase was detected. The Fe^{2+} content was particularly high in reactor I, in which the processes of recovery and leaching of sulfide minerals (including Fe^{2+}) occurred (Fig. 1a, curve *4*). Oxidation of Fe^{2+} occurred mainly in reactor II (Fig. 1b, curve *3*); in reactor III, iron was oxidized further (Fig. 1c, curve *3*); the amount of ferrous iron was insignificant, 0.10–0.35 g/l (Fig. 1c, curve *4*). Arsenic concentration in the reactors varied within the same range and reached the maximum of 2.25 g/l. In reactors I, II, and III, the average arsenic concentrations in the solution were 1.56, 1.72, and 1.29 g/l, respectively. A slight decrease in the iron and arsenic concentrations in the liquid phase of the pulp detected during the process can be attributed to precipitation of iron oxides, as well as to coprecipitation together with arsenic ions at high temperatures. The Eh values decreased slightly and then reverted back.

Analysis of the parameters of the liquid phase of the pulp during the oxidation of the three-component antimonite-containing substrate at 46 ± 1°C under the regime simulating imitating continuous cultivation revealed the following. The process, starting from reactor II, did not require pulp acidification. This suggests that conditions created in each of the serially connected reactors were specific; therefore the reactions of leaching/oxidation (or postleaching/postoxidation) were specific for these conditions as well. The decrease in the pH level to 1.4 and 1.3 in reactors II and III, respectively, indicates the high activity of sulfur-oxidizing bacteria (including antimonite-oxidizing microorganisms) in the community, since the Fe_S and As_S concentrations in the initial three-component mixture were not high (moderate). Importantly, the problems with jarosite passivation on the surfaces of oxidizable minerals are minimized at low pH. The regulation of the processes occurring in reactors II and III by the microbial community resulted in an increase in the Eh level as well.

Thus, in reactor I, the processes of mineral recovery and the initial stages of oxidation of leached ferrous iron, arsenopyrite, and antimonite were stable judging from the acidification kinetics of the pulp after 8-day cultivation. It can be assumed that, in the microbial community of this reactor, the microorganisms showing a certain substrate specificity (those oxidizing initial sulfide minerals) prevailed. Microorganisms showing high rates of both iron and reduced sulfur oxidation were probably predominant in the microbial community of reactor II. Reactor III contained the most acidified pulp (with pH of up to 1.3), the liquid phase of which virtually did not contain Fe^{+2}; an increase in the Fe^{3+} content was not detected as well. In this reactor, the microorganisms capable of active oxidation of reduced sulfur compounds (S^{2-} /S^0, polythionates, etc.) were probably predominant. The total arsenic content in the liquid phase of the pulp, the product of arsenopyrite oxidation, was essentially independent of the process stage.

The rate and extent of the oxidation of sulfide minerals by microbial association at 46°C was determined more precisely using the results of analysis of the ele-

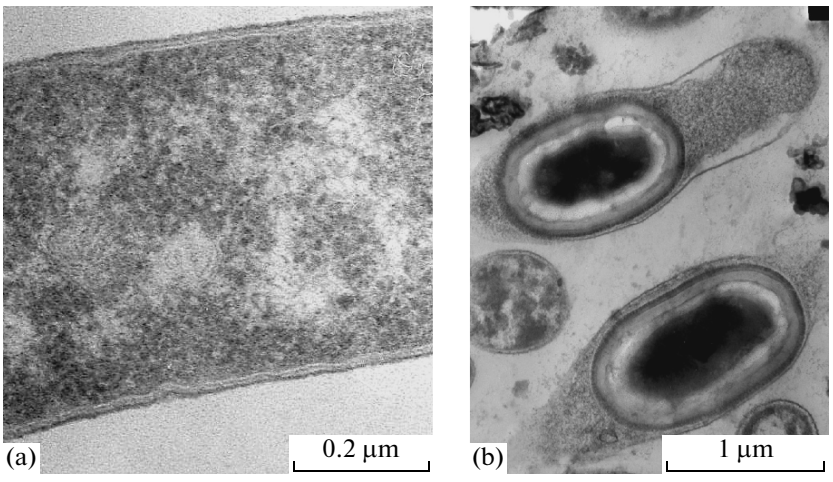

Fig. 2. Electron microphotographs of the cells of the microbial community involved in the processing of the pulp (16%) consisting of the mixture of high-antimony ore and ore concentrates under the semicontinuous mode (46 × 1°C) in the reactors: ultrathin sections of the cells of strain Sb-F (a); ultrathin sections of the sporulating cells of strain Sb-K (b).

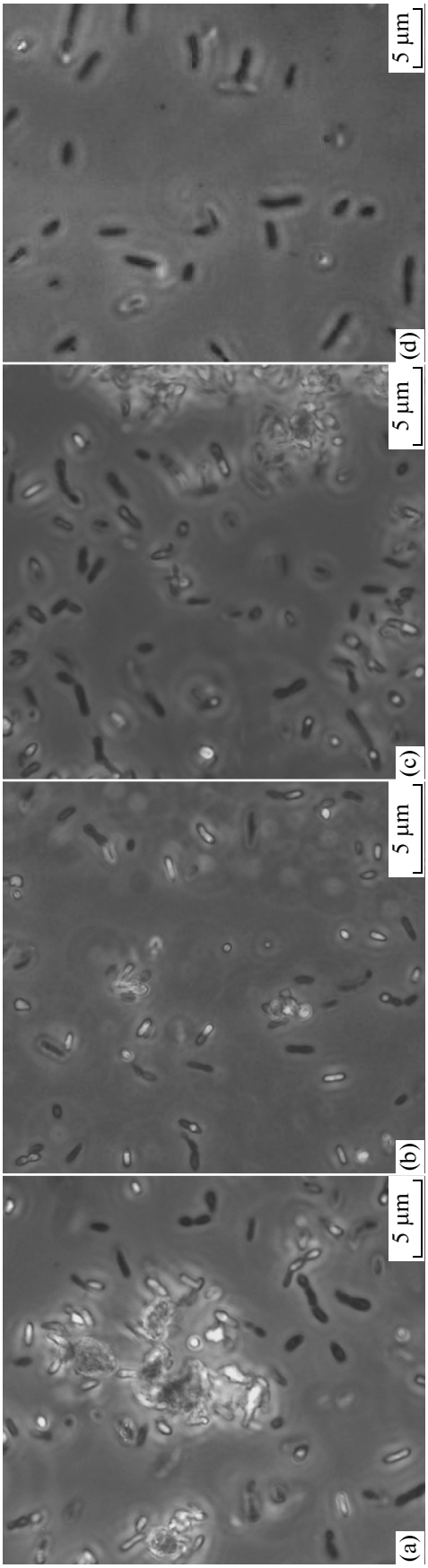

Fig. 3. Cell populations of the microbial community and isolates involved in the active oxidation of antimonite from the mixture of high-antimony ore and ore concentrates in the course of semicontinuous cultivation at 46 × 1°C. View of the aboriginal microbial community populations (a), Sb-S monoculture (b), Sb-K monoculture (c), and Sb-F monoculture (d). Light microscopy (phase contrast).

mental composition of biocakes and cyanidation. The degree of element oxidation in the course of the bacterial oxidation of antimonite and other sulfide minerals (considering the solid phase release) was 81.34, 98.17, and 91.22% for Sb_S, As_S, and Fe_S, respectively. The degree of gold extraction from the oxidation product (biocake) was 82.85%.

Isolation of the Members of the Thermophilic Microbial Community Oxidizing the Ore with High Antimony Content and Their Brief Description

Isolation of monocultures. Isolation of the pure cultures of thermoacidophilic bacteria dominating in the microbial community involved in the biooxidation of antimonite and other sulfide minerals at 46°C in the course of semicontinuous cultivation in reactors I, II, and III was carried out as described above. The strains were designated as follows: (i) Sb-K, the strain isolated

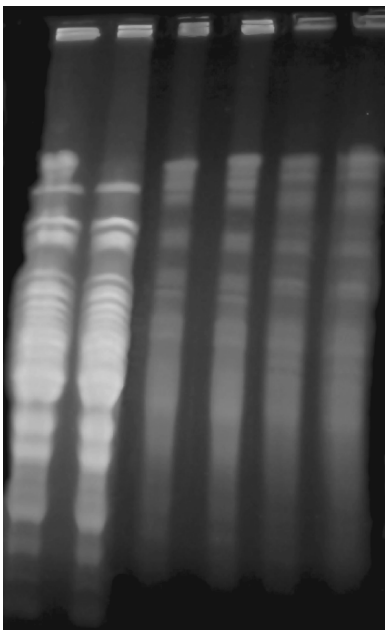

Fig. 4. Profiles of the *Not*I-cleaved chromosomal DNA fragments of the strains isolated from the pulp of ore concentrates and high-antimony ore from the Olympiadinskoe deposit and cultivated on various energy substrates: on the Fe^{2+}-containing medium (*1*), on the medium with ore and ore concentrates (*2*), and on the medium with S^0 (*3*). Pulsed-field electrophoresis conditions: voltage 13 V/cm, pulse time 10 s, temperature 15°C, duration 68 h.

Table 3. Growth rates of the strains isolated from the microbial association and the rates of oxidation of reduced iron and sulfur compounds under mixotrophic conditions at 46°C

Substrate	Strain Sb-S			Strain Sb-K			Strain Sb-F		
	μ_{max}, h^{-1}	Fe*	SO_4^{2-}**	μ_{max}, h^{-1}	Fe*	SO_4^{2-}**	μ_{max}, h^{-1}	Fe*	SO_4^{2-}**
Fe^{2+}	0.19	0.42	–	0.22	0.48	–	0.36	0.55	–
S^0	0.24	–	0.75	0.21	–	0.70	0.18	–	0.68
$S_2O_3^{2-}$	0.135	–	–	0.128	–	–	0.094	–	–
$S_4O_6^{2-}$	0.125	–	0.48	0.123	–	0.51	0.122	–	0.40
Sb_2S_3	0.045	–	0.37	0.053	–	0.42	0.047	–	0.39
Ore + concentrates	0.28	0.47	0.94	0.34	0.58	1.21	0.32	0.61	0.79

Note: The cultures were grown on the medium with 0.2 g/l yeast extract and Fe^{2+} or S^0 for 2 days, as well as with the sulfide minerals of high-antimony ore and flotation concentrates $S_2O_3^{2-}$ and $S_4O_6^{2-}$ for 3 days and with antimonite for 14 days. Initial concentration of sulfide minerals of the mixture or antimonite: 10 g/l; Fe^{2+}, S^0 (5 g/l); $S_4O_6^{2-}$, $S_2O_3^{2-}$ (10 mM). Peak rates of formation of terminal oxidation products: * Fe^{3+}, g/(l h) for experimental variants with Fe^{2+} and the sulfide minerals of ore and flotation concentrates; ** (SO_4^{2-}), g/(l day), for experimental variants with S^0 and sulfur-containing substrates; – not determined.

at the highest dilution on the medium (pH 1.7) with flotation concentrate and ore; (ii) Sb-F, the strain isolated at the highest dilution on the Fe^{2+}-containing medium (pH 1.8); and (iii) Sb-S, the strain dominating on the S_0-containing medium (pH 2.3). After the cultures were checked for purity by plating the heated spores onto agarose medium [20] and the application of the terminal dilution method, the isolates were grown under autotrophic, heterotrophic, and mixotrophic conditions, respectively, in the presence of only mineral substrates (S^0, Fe^{2+}), at high concentrations of yeast extract (1%) or mixotrophically. The results obtained revealed the phenotypic similarity between the isolates and bacteria of the genus *Sulfobacillus*: optimal growth was observed only in the latter case, i.e., in the presence of mineral substrates and yeast extract (0.02%). The isolates were not able to grow constantly under either lithotrophic or organotrophic conditions. No growth of any other microorganisms was detected, which confirmed the purity of the cultures obtained.

Morphology of the new strains. The exponential-phase cells of the isolates were straight or slightly curved rods with rounded ends. Cell polymorphism was a characteristic trait of these strains. The cells were nonmotile, with the exception of the cells of strain Sb-K, for which some motility, resembling positive chemotaxis, was detected. The strains stained gram-positive; their cell wall structure was of the gram-positive type, as can be clearly seen on ultrathin sections (Fig. 2a). The cells reproduced by binary division; chains consisting of 2–4 cells were detected. Spore formation was either terminal or subterminal (Fig. 2b). Figures 3a–3d show the general views of the microbial community and three isolates. The morphological properties, as well as the capacity for mixotrophic growth, of the isolates are similar to those of sulfobacilli.

Restriction analysis of the chromosomal DNA of the isolates. Figure 4 shows the results of the restriction analysis of the chromosomal DNA fragments of the tree isolates by pulsed-field gel electrophoresis in polyacrylamide gel [21]. It demonstrates that strains Sb-K, Sb-F, and Sb-S differ in terms of the number and size of the *Not*I-digested fragments of the chromosomal DNA; they represent taxonomically different bacterial cultures (Fig. 4, lanes *1–3*).

Growth under mixotrophic conditions. Earlier, we showed that three of the isolated cultures were capable of stable growth at peak rates only at the expense of mixotrophic nutrition, i.e., simultaneous utilization of inorganic and organic substrates (e.g., 0.02% yeast extract). S^0, Fe^{2+}, and sulfide minerals from the tree-component substrate mixture (antimony ore and flotation concentrates from the Olympiadinskoe deposit) were used as inorganic substrates.

Strain Sb-K, isolated from reactor I as the predominant strain on the medium with antimony ore and ore concentrates, grew well under mixotrophic conditions on media with all the aforementioned substrates. The highest growth rate (0.34 h^{-1}) was observed in the presence of metal sulfides (Table 3). Strain Sb-F, isolated from the microbial community of reactor II on the Fe^{2+}-containing medium as the predominant strain, grew in the medium with ferrous iron under mixotrophic conditions at the maximum specific rate of 0.36 h^{-1}. Strain Sb-S, isolated from the microbial community of reactor III as the predominant strain on

the sulfur-containing medium, grew at the expense of mixotrophic nutrition in the presence of elemental sulfur and sulfide minerals at specific rates of 0.24 and 0.28 h^{-1}, respectively.

Functional diversity and trophic functions of the isolates. Table 3 shows the specific growth rates and the rates of oxidation of mineral substrates by the isolates during growth under mixotrophic conditions on 9K medium with yeast extract, Fe^{2+}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, antimonite (Sb_2S_3), and the mixture of ore and flotation concentrates.

Strain Sb-S grew at the maximum specific growth rate (μ_{max}) on media with elemental sulfur and the mixture of the sulfide minerals of ore and ore concentrates (0.24 and 0.28 h^{-1}, respectively). The rate of oxidation of reduced sulfur to the terminal product (sulfate ion) was also high, 0.75 and 0.94 g/(l day), respectively. The cell yield on the sulfur-containing medium was 3.68×10^8/ml; on the medium with the sulfide minerals of ore and ore concentrates, it reached 3.21×10^8/ml. Despite the fact that the antimonite-containing medium was supplemented with 0.4 g/l Fe^{3+} in order to induce the processes of recovery/leaching and cofactor synthesis, the values of μ_{max} and cell yield reached 0.045 h^{-1} and 1.02×10^8/ml, respectively; the peak rate of antimonite oxidation was 0.37 g/(l day). On the antimonite-containing medium, a decrease in the cell size, cell sporulation, and the presence of refractory cells were observed at the end of growth, which can be attributed to the inhibitory effect of antimony on the growth processes. On media with other mineral substrates, the amounts of spores and refractory cells were lower. The growth rate of strain Sb-S on sulfur-containing substrates, such as tetrathionate and thiosulfate, the products of sulfur transformation, was higher than in the case of other strains [13, 17]. The levels of tetrathionate hydrolase and thiosulfate dehydrogenase activities of strain Sb-S were higher than those of other isolates by 20–30% (Tsaplina, I.A.; Bogdanova, T.I.; Sorokin, V.S.; Zhuravleva, A.E.; and Kondrat'eva, T.F., unpublished data). The strain isolated from the total microbial association as the microorganism prevailing during growth in the sulfur-containing medium was capable of switching from sulfur metabolism to iron oxidation. After the first transfer, it grew on the medium with ferrous iron at a specific rate of 0.19 h^{-1} and was capable of active iron oxidation at a peak rate of 0.42 g/(l h).

The results obtained suggest that oxidation of sulfur and the products of its transformation is the main function of strain Sb-S in the microbial community involved in the oxidation of antimonite-containing mixture of sulfide minerals under the continuous mode. However, the apparent universality of bacteria should be noted, since they fulfill various trophic functions (they are able to switch from sulfur metabolism to iron metabolism, as can be seen from the kinetic parameters of growth).

The best growth of strain Sb-K (0.34 h^{-1}) occurred in the medium containing ore with high antimony content and sulfide flotation concentrates as the main substrates (Table 3). After 24-h incubation, the number of cells reached its peak (3.45×10^8/ml) and remained stable for another 24 h. The culture was capable of active oxidation of both the iron and sulfur components of the substrates. The rate of oxidation of leached iron was 0.58 g/(l h); the rate of S^{2-}/S^0 oxidation was 1.21 g/(l day). Strain Sb-K was capable of active iron oxidation during growth on the Fe^{2+}-containing medium and was able to actively oxidize elemental sulfur on the S_0-containing medium (0.48 g/(l h) and 0.70 g/(l day), respectively). This strain was able to utilize other reduced sulfur compounds (antimonite, thiosulfate, and tetrathionate) as energy sources; the highest values of its kinetic parameters (as compared to other isolates) were detected during growth on the medium with Sb_2S_3.

Thus, it can be suggested that participation in the initial stages of processing (recovery of antimonite and metal sulfides, leaching of Fe^{2+}, Sb^{3+}, and As from sulfide minerals, and initiation of S^{2-}/S_0 and Fe^{2+} oxidation) is the main function of strain Sb-K in the microbial community involved in the oxidation of the mixture of antimonite-containing ore and sulfide concentrates.

The best growth ($\mu = 0.35$–0.36 h^{-1})of strain Sb-F isolated as the predominant strain on the Fe^{2+}-containing medium was observed during growth on the medium with this substrate which was oxidized at a rate of 0.55 g/(l h) (Table 3). The bacterial population was represented by morphologically different cells; small cells (as a result of nonuniform division) and refractory cells were present. However, the majority of cells in the population retained the morphological properties typical of this strain. After subculturing on the medium with elemental sulfur, the strain grew actively; it was able to switch to another type of metabolism, to grow, and to oxidize the substrate at the rates of 0.18 h^{-1} and 0.68 g/(l day), respectively. The culture actively oxidized the mixture of sulfide minerals and utilized other substrates, including antimonite, as energy sources. On the antimonite-containing medium, spore formation and transformation of active cells into refractory cells was observed in a part of the population.

The kinetic parameters measured during growth of strain Sb-F on various substrates, as well as the oxidative activity of this strain isolated from reactor II, where it predominated, suggest that oxidation of ferrous iron and postleaching of metals is the main function of this strain in the microbial community involved in continuous oxidation of the antimonite-containing mixture of sulfide minerals; oxidation of reduced sulfur is its secondary function.

Thus, the final stage of our investigation has demonstrated different and identical phenotypic characteristics of three new cultures growing under mix-

otrophic conditions. All three strains have a certain mechanism that helps them to rapidly switch from iron metabolism to sulfur metabolism and vice versa. Therefore, these stains are able to both augment each other's functions and dominate under certain trophic conditions. The strategy of isolation of the predominant culture from the microbial population involved in the processing of sulfide minerals was based on the ability of this culture to possess the highest rates of growth and oxidation of a certain substrate added to the medium. This strategy supported the growth strategy utilized by the microbial association in the course of continuous cultivation.

Importantly, all three strains were found to be able to grow and oxidize antimonite. Their oxidative effect was probably additive in the dense pulp. The high degree of sulfide antimony oxidation under the semicontinuous regime of oxidation of antimonite-containing ores by the microbial community including the "sulfobacilli" cultures with pronounced substrate specificity suggests the possibility of applying the developed method for the removal of antimonite from initial gold-containing concentrates. This was confirmed by the rate of gold extraction from biocake. Further detailed investigation of the effect of antimony, belonging to the group of the so-called thiol toxins [19], on the physiological state of the isolated bacteria is required.

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