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EXPERIMENTAL ARTICLES

Response to Oxygen Limitation in Bacteria of the Genus *Sulfobacillus*

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Abstract—For cultures of moderately thermophilic chemolithotrophic bacteria *Sulfobacillus sibiricus* N1 and SSO, *S. thermosulfidooxidans* subsp. *asporogenes* 41, and the thermotolerant strain *S. thermotolerans* Kr1 grown under forced aeration and in a high medium layer without aeration, growth characteristics, substrate consumption, and exometabolite formation were compared. Sulfobacilli grown under oxygen limitation exhibited greater generation time, longer growth period, cell yield decreased by from 40 to 85% (depending on the strain), suppressed cell respiration (demonstrated for *S. sibiricus* N1), accumulation of exometabolites (acetate and propionate) in the medium, and emergence of resting forms. For strains N1, SSO, and Kr1, oscillations of Fe(II) and Fe(III) content in the medium were revealed. For *S. sibiricus* N1 and *S. thermotolerans* Kr1, grown under hypoxia (0.07% O_2 in the gas phase), coupling of substrate oxidation with Fe(III) reduction was revealed, as well as utilization of Fe(III) as an electron acceptor alternative to oxygen. The role of labile energy and constructive metabolism for survival of sulfobacilli under diverse conditions is discussed.

Key words: aeration conditions, growth, substrates, morphology, respiration, iron oxidoreduction.

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Microorganisms of the genus *Sulfobacillus* are a well-defined group of thermophilic chemolithotrophic bacteria [1]. They inhabit deposits of sulfide ores and minerals in various geographical locations, open mines of developed deposits, self-heating piles of coal, ores, etc. In such ecosystems, limitation in certain substrates may occur due to inconstant flows of water, dissolved gases, and organic matter, as well as temperature increase resulting from the oxidation of reduced sulfur compounds. Even in aerated reactors, biological oxidation of sulfide ore concentrates results in formation of local anoxygenic microzones where the passivation layers are formed (containing S^0, jarosites, and other precipitates), which prevent microbial opening and oxidation of ores. The organisms encounter stress factors, including fluctuating oxygen concentrations. The states of normoxia (aerobiosis), hypoxia (microaerobiosis), and anoxia (anaerobiosis) may occur sequentially. Sulfobacilli had to develop adaptive mechanisms of metabolic regulation in response to such changes in their habitats.

Bacteria of the genus *Sulfobacillus* are known to have flexible facultatively chemolithoautotrophic aerobic metabolism; they possess a broad spectrum of carboxylation enzymes for auto- and heterotrophic CO_2 fixation [2–7]. They are not obligate aerobes [8]; a number of thermophilic sulfobacilli strains were shown to grow mixotrophically in the absence of air bubbling [9, 10] or organotrophically under anoxic conditions on media with glycerol and yeast extract, or lithotrophically on media with tetrathionate. Energy for growth is stored due to the coupling of the oxidation of these substrates and Fe(III) reduction to Fe(II) [9, 11].

In cell-free extracts of two *Sulfobacillus sibiricus* strains, N1 and SSO, grown mixotrophically under air oxygen partial pressure in a high layer of the medium containing reduced species of sulfur or iron, S^0, and SO_3^{2-}:Fe(III) oxidoreductases were detected [10]. Such enzymes, which are able to use Fe(III) as the sole electron acceptor, reducing it to Fe(II) in the course of sulfur or sulfite oxidation, have been previously found in *Acidithiobacillus ferrooxidans* [12, 13].

The goal of the present work was to determine the effect of oxygen regimes of cultivation on growth, respiration rate, and oxidative activity of some bacteria of the genus *Sulfobacillus, S. sibiricus, S. thermosulfidooxidans* subsp. *asporogenes*, and *S. thermotolerans*.

MATERIALS AND METHODS

Subjects of research. Three strains of moderately thermophilic acidophilic bacteria were used: two

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S. sibiricus strains, N1 and SSO; *S. thermosulfidooxidans* subsp. *asporogenes* 41, as well as one thermotolerant strain *S. thermotolerans* Kr1.

Strain N1^T (VKM B-2380^T = DSM 17363^T) and strain Kr1^T (VKM B-2339^T = DSM 17362^T) were isolated from dense pulp in the course of industrial trials of the technology for processing of, respectively, the gold-containing pyrite–arsenopyrite concentrate of the Nezhdaninskoe deposit (Sakha Republic) [14] and the pyrrhotite pyrite–arsenopyrite ore concentrate of the Olympiadinskoe deposit (East Siberia) [15]. Strain SSO was isolated from the heating zone of an open mine of the Olympiadinskoe deposit of the above ore [7, 16]. The asporogenic strain 41 (Institute of Microbiology, Armenia B-6981^T) was isolated from mine water of the pile of the Armanis multimetallic complex ore sulfide deposit [17].

Cultivation Strains N1, SSO, and Kr1 were grown in 9K medium [18] containing the following (g/l): $(NH_4)_2SO_4$, 3.0; KCl, 0.1; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 20–100 mM (as Fe(II)); glucose, 1.1 mM; yeast extract, 0.2; and $Na_2S_2O_3$, 2 mM. Strain 41 was cultivated in the Brierly medium [19] containing the following (g/l): $(NH_4)_2SO_4$, 0.5; KCl, 0.2; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 60 mM (as Fe(II)); and glucose, 2.75 mM. The pH value was adjusted at 1.6–1.8 with 10 N H_2SO_4. The mineral base was autoclaved at 1 atm. Yeast extract, glucose, glycerol, and tetrathionate were sterilized separately at 0.5 atm. Solutions of Fe(II) and Fe(III) were sterilized by filtration and added aseptically to sterile media. The inoculum was grown on the medium with Fe(II) and yeast extract; its amount for aerobic conditions and hypoxia was 10 and 20 vol %, respectively. Moderately thermophilic and thermotolerant strains were cultivated at 55 and 40°C, respectively. For inoculum, all strains were grown on a shaker (180 rpm) in 250-ml flasks with 100-ml medium at the $V_{flask} : V_{medium}$ ratio of 2.5 : 1. For some experiments at air partial pressure (21% oxygen in the gas phase) with strain *S. sibiricus* N1, the culture was grown for ten transfers under static conditions in a high layer of the medium with ferrous iron ($V_{flask} : V_{medium} = 1.4 : 1$).

Further cultivation was carried out in various regimes and in various vessels: (1) without forced aeration, at the partial pressure of oxygen in air (21% in the gas phase), in bottles with $V_{bottle} : V_{medium} = 1.2–1.4 : 1$; (2) at fixed oxygen content in the gas phase (15–17%) in Hungate tubes; and (3) at fixed oxygen content in the gas phase (0.07%) in vials, i.e., under hypoxia close to anoxia. Intense aeration by blowing 1 volume of air per 1 volume of medium per minute in 5-l bottles with $V_{bottle} : V_{medium} = 1.7 : 1$ was used as the control.

The medium was swirled after inoculation and prior to sampling in order to increase diffusion flows, improve oxygen and CO_2 supply to the cells, and average out the results of analyses.

Experiments with the first aeration regime were carried out with all the strains under mixotrophic conditions on media with Fe(II), ($S_2O_3^{2-}$ when mentioned), glucose, and yeast extract (strain 41, without yeast extract). The second aeration regime was studied on mixotrophically and organotrophically growing *S. sibiricus* N1. The third regime was studied on *S. sibiricus* N1 and *S. thermotolerans* Kr1. In the latter case, several variants of the media were used, containing yeast extract (0.02%), glycerol (10 mM), or tetrathionate (3 mM). The volume of Hungate tubes was 18 ml; they contained 15 ml of medium (including inoculum). The medium was boiled, sealed, cooled, bubbled with CO_2 to provide dissolved carbon dioxide (considering the diversity of carboxylases), and sterilized. Inoculum, thiosulfate, and 7.5 mM Fe(III) were added aseptically with a syringe; variants with 15–17% O_2 in the gas phase were selected. For microaerobic cultivation (third aeration regime), 40-ml vials containing 23 ml of the medium (including inoculum) were used. As an electron acceptor, 10 mM Fe(III) was used. The medium containing an organic or inorganic substrate was boiled, cooled, and dispensed under nitrogen flow. The vials were sealed with rubber stoppers and metal caps and bubbled with CO_2 for 5–7 min; the inoculum and Fe(III) were added aseptically after sterilization.

Inocula and samples were examined for the presence of contaminating microorganisms by light microscopy and plating on agarose media [20]. Controls without inoculation and with the cells killed by heating were used.

Analytical methods. *Cell concentrations* were determined by phase contrast microscopy (Lumam I1, LOMO, Russia). Generation time, rates of substrate utilization, and concentrations of glucose and iron ions were determined and calculated as described previously [4]. The correlation between cell yield and iron reduction was analyzed by direct cell count and determination of the concentrations of iron species.

Oxygen concentration in the gas phase was determined on a Khromatek Kristal 5000.1 gas chromatograph (Russia). Oxygen content (%) was calculated from the peak area, assuming 21% concentration of O_2 in air.

For determination of the *respiration rate*, at the late-exponential growth phase, cells were collected by centrifugation (4500 *g*) at room temperature, washed with the medium without energy sources (pH 1.7), and incubated in the same medium at 55°C on a rotor shaker (180 rpm) in order to decrease endogenic respiration.

Consumption of O_2 by suspensions of sulfobacilli cells was measured on an LP-9 polarograph (Laboratorni pristroje, Czech Republic) with a Clark-type electrode and a silver chloride electrode as a nonpolarizing reference electrode. The volume of the polarographic cell was 1 ml; the measurements were taken at room temperature. The reaction mixture contained

Table 1. Growth parameters of *S. sibiricus* N1, *S. sibiricus* SSO, *S. thermosulfidooxidans* subsp. *asporogenes* 41, and *S. thermotolerans* Kr1 depending on the oxygen regime of cultivation

Strain	Lag phase duration, h	Minimal generation time, h	Growth duration, h	Cell yield, 1×10^7/ml
		O_2 partial pressure		
N1 (first transfer)	15–20	10	150–160	12.9
N1 (11th transfer)	28–30	20	200–210	8.6
SSO	16–20	11	130–150	10.1
41	50–80	46	250–260	0.9
Kr1	15–18	14	150–160	15.4
		Intense aeration		
N1	5	1.8	12–14	21.5
SSO	4	2.3	13–15	18.4
41	3.5	3.0	16–18	7.1
Kr1	5	2.3	15–16	22.3

the cell suspension in 9K medium, pH 1.7 and various substrates: $FeSO_4 \cdot 7H_2O$ (40 mM as Fe^{2+}), yeast extract (0.03%), or glucose (2 mM). The respiration rate in nmol O_2/(min mg protein) was calculated relative to the rate of oxygen binding by thiosulfate (250 nmol) dissolved in 1 ml of water at room temperature.

Exometabolites, the products of metabolism at the end of bacterial growth, were analyzed by two methods. For gas–liquid chromatography, Gas-Khrom 3700 (Russia) was used, equipped with a flame ionization detector and 1.5-m Sovpol column, at the column temperature of 190°C; argon was used as a carrier gas. Thin-layer chromatography on Silufol was carried out in the *n*-amyl alcohol : formic acid (1 : 1) solvent system. Prior to analysis, the samples were alkalinized to pH 9.0 and evaporated to a small volume; inorganic salts were precipitated, and the sample was centrifuged. After chromatography, organic acids were revealed with bromothymol blue. Keto acids were also determined by thin-layer chromatography of their derivatives as 2,4-dinitrophenyl hydrazones [21].

All experiments, including the analytical measurements, were performed in three or four repeats. Statistically reliable differences at $p < 0.05$ are discussed.

RESULTS

Effect of oxygen on growth processes and cell yield. Experimental results on the effect of different oxygen concentrations on cell yield of sulfobacilli cultures are presented in Table 1. It can be seen that the cultures survive oxygen deficiency in the medium. For mixotrophic metabolism, cell yields for intense aeration and for air partial oxygen pressure were similar, about 50–60% (less than 20% for strain 41) of the maximum obtained by bubbling the medium with air (the control, $V_{bottle} : V_{medium} = 1.7 : 1$). In the 11th transfer of *S. sibiricus* N1 adapted to oxygen limitation, cell yield was 40% of the maximum. Prolonged adaptation of strain N1 resulted in increased duration of culture growth.

Lag phase duration was 3.5–5 h in the control (Table 1); in the experiment ($V_{bottle} : V_{medium} = 1.4 : 1$), for strains *S. sibiricus* N1 and SSO and *S. thermotolerans* Kr1, it was 15–20 h and 30 h (for the first and 11th transfers, respectively), 16–20 h, and 15–18 h, respectively. For strain *S. thermosulfidooxidans* subsp. *asporogenes* 41 grown without forced aeration ($V_{flask} : V_{medium} = 1.25 : 1$) on the medium without yeast extract and with the nitrogen concentration six times lower than for other sulfobacilli, the lag phase was very long; no changes in the concentration of medium components and in cell numbers occurred for 50–80 h. During the growth phase, the minimal generation time for strains N1, SSO, and Kr1 was 10 (20 in the 11th transfer), 11, and 14 h, respectively; for strain 41, it was 46 h. Growth duration for the first three strains was within 130–160 h (200–210 h for the 11th transfer of strain N1); for strain 41, it was 250–260 h. The average cell yield for strain N1 was 12.9×10^7 cells/ml (8.6×10^7 in the 11th transfer); for strains SSO, 41, and Kr1, the yields were 10.1×10^7, 0.9×10^7, and 15.4×10^7 cells/ml, respectively. In the control (in complete media without oxygen limitation), strains N1, SSO, 41, and Kr1 had a generation time of 1.8–3.0 h and cell yield of 21.5×10^7, 18.4×10^7, 7.1×10^7, and 22.3×10^7 cells/ml, respectively.

In Hungate tubes with decreased O_2 content in the gas phase (15–17%, second regime), cell yield of strain *S. sibiricus* N1 was approximately 50% of the level obtained under intense aeration. Growth was best in the medium supplemented with yeast extract, thiosulfate, and Fe(III). With glycerol as the electron donor, growth was less intense (Table 2). Organotrophic growth of *S. sibiricus* strain N1 in the vials

Table 2. Utilization of Fe(III) as an alternative electron acceptor by strains *S. sibiricus* N1 and *S. thermotolerans* Kr1 at decreased O_2 content in the gas phase and under hypoxia

Substrates and cultivation conditions		Cell number, 1×10^7/ml	Fe(III), mM	Fe(II), mM
S. sibiricus N1				
Yeast extract, 0.02% + thiosulfate, 3 mM	17% O_2 in the gas phase	11.2	1.25	5.75
Glycerol, 7.5 mM	15% O_2 in the gas phase	10.0	3.2	2.2
Glycerol, 10.0 mM	0.07% O_2 in the gas phase (hypoxia close to anoxia)	9.1	0	10.0
S. thermotolerans Kr1				
Yeast extract, 0.02%	0.07% O_2 in the gas phase (hypoxia close to anoxia)	8.7	0	10.0
Glycerol, 10.0 mM	0.07% O_2 in the gas phase (hypoxia close to anoxia)	8.3	0	10.0

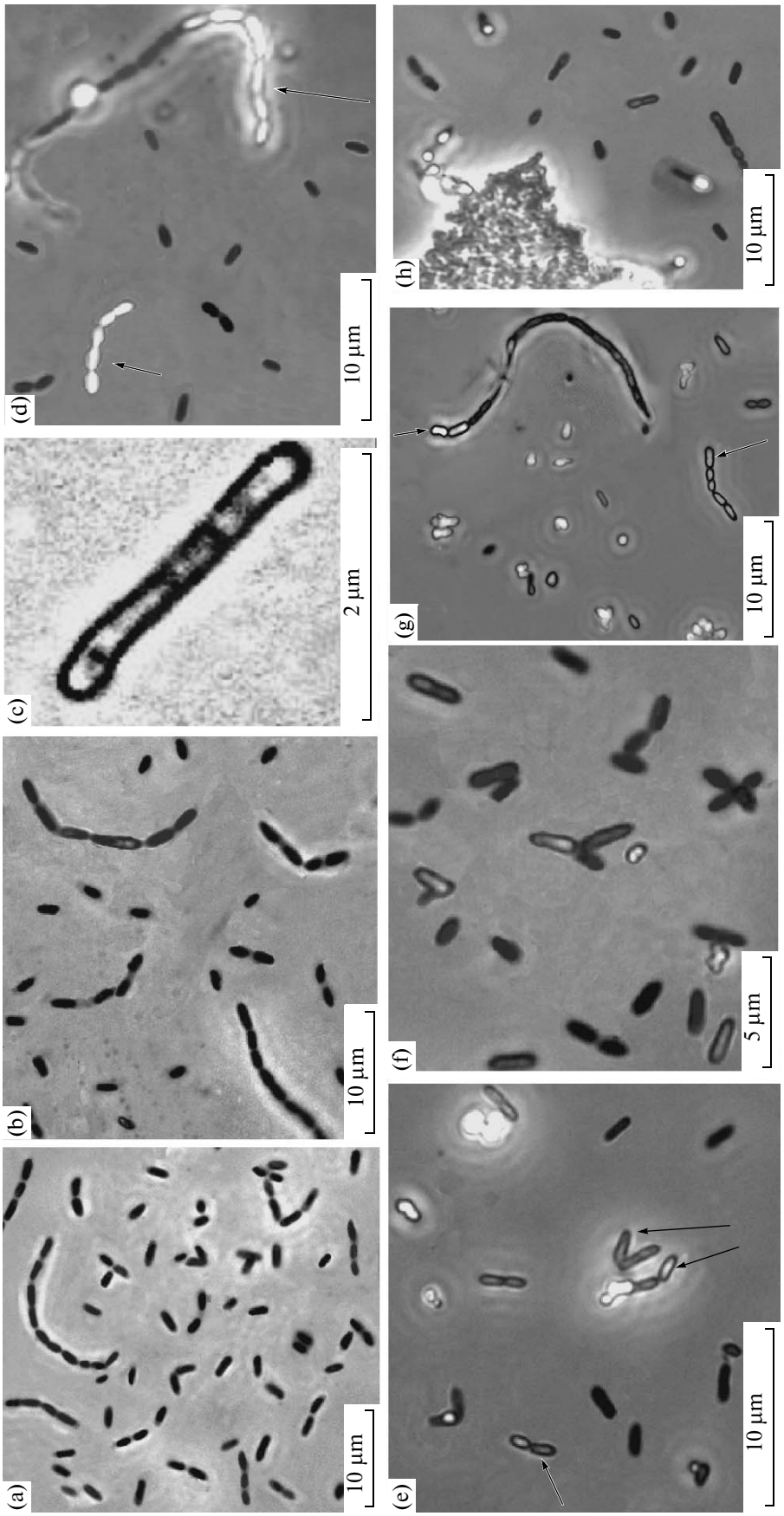
Note: Inoculum, 1.0×10^7 cells/ml.

under hypoxia (0.07% O_2 in the gas phase, third regime) with yeast extract or glycerol resulted in similar cell numbers (Table 2). Microaerobic cultivation of *S. thermotolerans* Kr1 yielded similar results. Thus, after 8 days of cultivation on media with these substrates, growth under hypoxia close to anaerobic conditions was not much worse than organotrophic growth of these cultures on media with glucose and yeast extract under intense aeration [7]. This finding confirms the possibility of facultative growth of sulfobacilli at very low oxygen concentrations. Since on media with tetrathionate the number of vegetative cells was about 20% of the total cell number, both cultures exhibited only sustaining metabolism due to utilization of intracellular reserves.

Microscopic examination revealed that the cells cultivated in bottles with a high layer of medium (i.e., under limitation by dissolved O_2) aggregated with formation of a mucous suspension. Due to periodical swirling resulting from sampling, the cells were moved to the zone of optimal conditions located close to the surface of the medium (Fig. 1a). Single cells or cell chains able to overcome diffusion limitation by the substrates, oxygen, and CO_2 were distributed in the medium column as well (Fig. 1b). Cultivation of sulfobacilli under growth-limiting conditions resulted in the emergence of sporulating cells, spores, and cells with semitransparent content, probably refractory (resting forms). Depending on the strain and the number of transfers, the latter ones constituted 5–30% of the total cell number. One such cell from the culture of *S. thermosulfidooxidans* subsp. *asporogenes* is shown on Fig. 1c. Figures 1d and 1e show the populations of *S. sibiricus* SSO and N1 (11th transfer), respectively, at the end of growth. Cells can be seen that differ in size and refractive capacity from the vegetative cells. When these cultures were transferred to static conditions (thin layer of medium, no active aeration), neither semitransparent nor lysing cells were observed; the populations contained regular vegetative forms. The number of refractive cells increased under extreme conditions of oxygen limitation, at numerous transfers under oxygen deficiency, as was demonstrated for *S. sibiricus* N1 (Fig. 1e), and under hypoxia, as was demonstrated for *S. sibiricus* N1 and *S. thermotolerans* Kr1 (Fig. 1f). For example, the number of resting forms may reach 80% under microaerobic conditions in the medium with tetrathionate (Fig. 1g). Refractory cells and spores were present in the population; however, most of the cells divided (not more than twice). The cells remained viable at subsequent transfers. The population of *S. sibiricus* N1 grown in Hungate tubes (second oxygen regime) contained mostly dividing single cells or chains of several cells; sporulating and refractory forms, which could form aggregates, were a minor fraction (Fig. 1h).

Efficiency of substrate utilization under oxygen deficiency. Oxidoreduction of iron. Sulfobacilli strains exhibit high rates of Fe(II) oxidation under intense aeration. The maximal iron reduction rate for growing cells of *S. sibiricus* N1 and SSO, *S. thermosulfidooxidans* subsp. *asporogenes* 41, and *S. thermotolerans* Kr1

Fig. 1. Morphology of sulfobacilli cells under oxygen limitation. First transfer at atmospheric partial pressure: *S. sibiricus* N1 cells in the surface layer (a), *S. sibiricus* SSO cells in depth (b); refractive cell of *S. thermosulfidooxidans* subsp. *asporogenes* 41 in the medium depth (c), cell populations of *S. sibiricus* SSO (d) and 11th transfer of *S. sibiricus* N1 (e) at the end of cultivation (arrows indicate refractive cells), *S. thermotolerans* Kr1 cells grown under hypoxia with yeast extract (f) and tetrathionate (g), and *S. sibiricus* N1 population at the end of cultivation in Hungate tubes on medium with glycerol at fixed O_2 content in the gas phase (h). Scale bar, 10 μm (a, b, d, g, h, e), 2 μm (c), and 5 μm (f). Light microscopy, phase contrast.


(a)
10 μm
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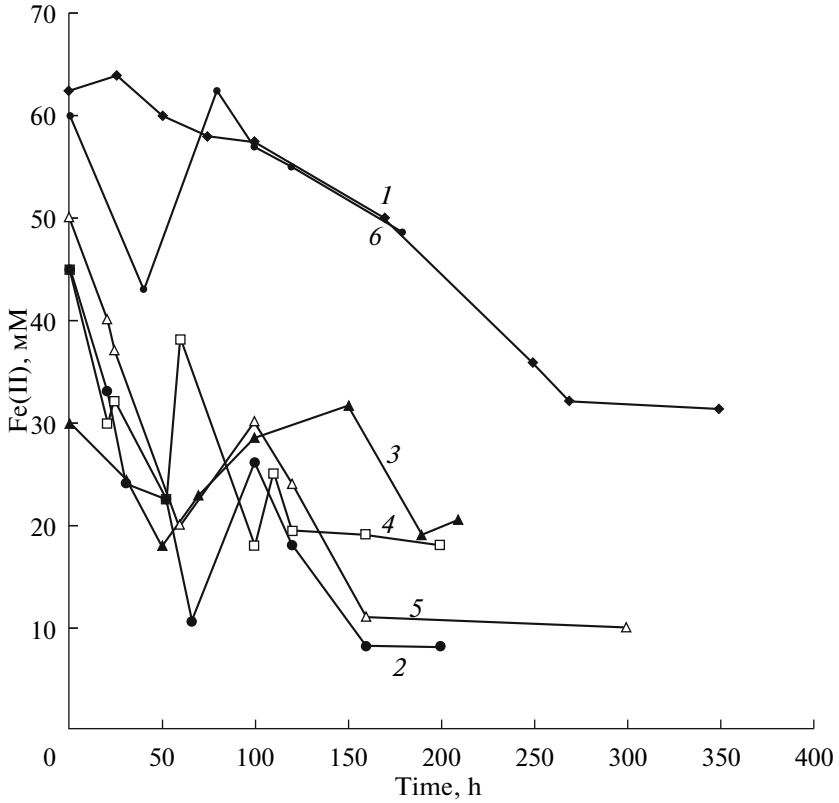

Fig. 2. Iron oxidoreduction by thermophilic sulfobacilli strains growing without intense aeration; (first oxygen regime, $V_{bottle}/V_{medium} = 1.2–1.4 : 1$) in a high layer of medium containing ferrous sulfate, yeast extract, and glucose, supplemented with thiosulfate. Cultivation temperature 55°C for *S. sibiricus* strains N1 and SSO and *S. thermotolerans* subsp. *asporogenes* 41 (medium without yeast extract) and 40°C for *S. thermotolerans* Kr1. Strain 41 (*1*); strain N1, first transfer (*2*); strain N1, first transfer (*3*); strain SSO 11th (*4*); strain Kr1 (*5*), and strain N1, tenth transfer, flask (*6*).

was 7.15, 3.2, 6.2, and 5.8 mM $h^{-1}l^{-1}$, respectively. In the course of growth, 45, 50, 40, and 60% of glucose was utilized by strains N1, SSO, 41, and Kr1, respectively. At the end of cultivation, no extracellular metabolites were revealed in the culture liquid by gas and thin-layer chromatography.

As was mentioned earlier, the cultures grown in a dynamic mode on a shaker (180 rpm) were used as inocula in the investigation of the limiting effect of oxygen on bacteria cultivated without intense aeration. Strain *S. sibiricus* N1 was gradually adapted to oxygen deficiency by sequential transfers in flasks with a high medium layer ($V_{flask} : V_{medium} = 1.4 : 1$) without shaking; it was subsequently also used to inoculate the bottles. In the course of preparation of such inocula, a gradual increase in the concentration of unoxidized Fe(II) was observed. For example, while strain N1 oxidized completely 100 mM Fe(II) during the first five transfers, approximately 5% and approximately 25–30% ferrous iron remained in the medium by the end of growth in the 6th and 10th transfer, respectively; in the latter case, the initial Fe(II) concentration was 60 mM. Analysis of the samples collected in the course of cultivation in flasks revealed fluctuations in the content of iron species in the last variant. Iron oxidation was followed by iron reduction. For example, Fe(II) oxidation occurred during 40 h after inoculation and was followed by the phase of Fe(III) reduction. Due to the introduction of unoxidized iron with the inoculum, Fe(II) concentrations were somewhat higher than the initial ones. Ferrous iron was then slowly oxidized.

After addition of the inoculum (N1 cells grown under dynamic conditions) into a bottle with high level of the medium, iron oxidation to the level of 10.5 mM was initially observed (Fig. 2, curve *2*). Glucose was consumed. The phase of iron reduction followed, when Fe(II) increased and glucose consumption continued to 30% of its initial amount. Then the cells switched to oxidation of ferrous iron; glucose was utilized, albeit at lower rates. By the end of the experiment, approximately 8 mM Fe(II) and 0.65 mM glucose (60% of the initial concentration) remained in the medium. Exometabolites, acetate and propionate (0.97 and 1.13 mg/l, respectively), were present in the medium. Formate, keto acids, and alcohols were not detected.

Similar patterns of iron oxidoreduction were observed in the experiments under atmospheric partial pressure of oxygen, with the culture grown in flasks

with a high layer of medium for ten transfers used as inoculum (Fig. 2, curve *3*, 11th transfer, bottle). Adaptation of the sulfobacilli to growth under oxygen deficiency resulted in increased growth time, decreased cell yield, and increased degree of glucose utilization (to 50% of the initial amount). The content of extracellular metabolites, propionate and acetate, was 0.62 and 0.65 mg/l, respectively.

Experiments with strains SSO and Kr1 confirmed iron oxidoreduction by the cultures growing under atmospheric partial oxygen pressure (Fig. 2, curves *4* and *5*). By the end of growth, glucose consumption was approximately 40 and 50%, respectively. In the culture liquid of strains SSO and Kr1, the same extracellular metabolites were detected, propionate (0.70 and 0.85 mg/l) and acetate (0.53 and 0.9 mg/l), respectively.

In experiments with *S. thermosulfidooxidans* subsp. *asporogenes* 41 growing without air bubbling (first regime), the population developed due to iron oxidation (Fig. 2, curve *1*) and glucose utilization. The rates of these processes were low. The maximal rate of Fe(II) oxidation was 0.18 mM $h^{-1}l^{-1}$, while for strains Kr1, N1, and SSO growing under similar conditions these rates were 0.70, 0.75, and 0.59 mM $h^{-1}l^{-1}$, respectively. After 270–300 h of cultivation, 50% of the initial Fe(II) and 60% of glucose remained in the medium. Exometabolites, acetate (0.6 mg/l) and traces of propionate, were also present. Under O_2 deficiency, only weak fluctuations of the concentrations of ferric and ferrous iron were observed.

Iron reduction did not occur in the control with sterile media distributed into bottles in a high layer; chemical oxidation of Fe(II) was low.

Results of analysis of iron species and O_2 concentrations in the gas phase after 8 days of cultivation of *S. sibiricus* N1 in Hungate tubes (second oxygen regime) under fixed initial oxygen content in the gas phase (15–17%) are presented in Table 2. The medium containing yeast extract and thiosulfate provided the best results in terms of culture growth; in acidic medium thiosulfate was converted to sulfur and sulfite similarly to [22]. In this variant, oxygen consumption by the cells was observed; oxygen content in the gas phase decreased from 17.0 to 10.3%. Ferric iron was reduced; it was probably utilized as a second electron acceptor. By the end of the experiment, out of the total 7.5 mM Fe(III) 1.25 mM Fe(III) and 5.75 mM Fe(II) were detected in the medium; the remaining iron was in the precipitate, similarly to the control. In the case of organotrophic growth on glycerol, strain N1 utilized oxygen of the gas phase to a greater degree than in other variants; after 8 days of cultivation, O_2 concentration decreased from 15.5 to 5.6%. The content of Fe(III) and Fe(II) in the medium was 3.2 and 2.2 mM, respectively; the control and precipitate contained approximately 2 mM. Thus, at fixed oxygen content in the gas phase, oxidation of growth substrates could involve utilization of Fe(III) as an additional electron acceptor, in spite of the presence of oxygen in the gas phase (27 and 49% of the initial O_2 content).

The results of cultivation of the moderately thermophilic *S. sibiricus* N1 and the thermotolerant *S. thermotolerans* Kr1 under microaerobic conditions (third oxygen regime) are presented in Table 2. Under harsh oxygen deficiency, 10 mM Fe(III) was completely reduced to Fe(II), thus demonstrating the possibility of using ferric iron as an oxidizer for energy supply of cell growth by coupling these processes. Glycerol and yeast extract were used as substrates. In the control (sterile cell-free and with the cells killed by heating), ferrous iron was not detected; Fe(III) was revealed in the precipitate. Therefore iron reduction in the experimental variants is probably not caused by reducing components of the inoculum or of the medium. Although a certain increase in the cell number was observed in the variant with tetrathionate after 8 days of cultivation, sustaining metabolism probably takes place due to utilization of intracellular reserves. Fe(III) did not occur.

Respiration of strain N1 cell suspensions. Experiments with *S. sibiricus* N1 were carried out in order to compare the viability and activity of sulfobacillar cultures under different oxygen concentrations in the medium, in a high layer of medium without aeration (first transfer) and under intense aeration (control). Respiration rates of bacterial suspensions were measured in a polarographic cell; capacity for active growth and substrate oxidation after subsequent transfer to shaking flasks was also determined.

Addition of Fe(II) to the incubation medium (salt base of 9K medium) with strain N1 cell suspension grown under oxygen limitation resulted in the rate of oxygen consumption 457 nmol O_2/(min mg protein). On the medium with glucose or yeast extract as an energy source, the respiration rates were 353.6 and 254 nmol O_2/(min mg protein), respectively. The level of endogenic respiration was zero.

The respiration rates of cell suspensions grown under intense aeration (after deduction of the endogenic level) after addition of Fe(II), glucose, or yeast extract to the incubation mixture were 520, 220, and 295.5 nmol O_2/(min mg protein), respectively.

Thus, the respiration rates of the cells grown in a high layer of medium at atmospheric oxygen partial pressure (without control of aeration) and of the cells grown under intense aeration did not differ significantly. The cells grown under intense aeration had higher respiration rate on Fe(II) and similar respiration rates on yeast extract, while the cells grown under diffusion limitation of oxygen input exhibited higher respiration rates on glucose.

Transfer of *S. sibiricus* N1 culture grown at O_2 partial pressure into shaking flasks revealed highly active iron oxidation and capacity for glucose assimilation.

The values were close to those cited above for the cultures grown in well-aerated media.

In subsequent experiments, the regulatory effect of oxygen on the pathways of carbon metabolism and the enzymatic activity of three moderately thermophilic sulfobacilli was studied at the subcellular level [23].

DISCUSSION

The results of our investigation confirmed that members of the genus *Sulfobacillus*, which are facultative anaerobes according to the amended phenotypic description [8], are capable of iron reduction on various substrates.

Dissimilatory Fe(III) reduction is well known for sulfur- and iron-oxidizing bacteria grown under anaerobic conditions, with organic and inorganic substrates such as glycerol, elemental sulfur, tetrathionate, chalcopyrite, etc., as electron donors [9, 24, 25]. This feature has been described for gram-positive and gram-negative bacteria and for archaea. Reduction of Fe(III) to Fe(II) by extreme acidophiles was observed both under oxygen-free conditions and under limited aeration, when the content of dissolved oxygen decreased to 40–60% of the initial maximum value [26].

In the present work, iron oxidoreduction was demonstrated for three strains of thermoacidophilic sulfobacilli, *S. sibiricus* N1 and SSO and *S. thermotolerans* Kr1. This process did not require strictly anaerobic conditions and occurred in bacterial cultures growing in a high layer of medium at a decreased rate of oxygen diffusion. In the course of iron oxidoreduction, metabolism could change from mixotrophic, characteristic of the stage of active Fe(II) oxidation and assimilation of glucose (probably of yeast extract as well) with oxygen as an electron acceptor, to organoheterotrophic, when consumption of organic substrates was accompanied by Fe(III) reduction to Fe(II). This process of iron oxidoreduction is similar to the reaction of iron oxidation–reduction in three *S. acidophilus* strains, strain *S. thermosulfidooxidans* TH1, and strain *Acidithiobacillus ferrooxidans* TH3 grown under the same conditions [9]. In our experiments, metabolism of sulfobacilli under oxygen deficiency probably remained one of a mixed type during Fe(III) reduction due to utilization, apart from organic compounds, of elemental sulfur and sulfite formed from thiosulfate in the medium [10].

In the present work, only one culture, *S. thermosulfidooxidans* subsp. *asporogenes* 41, did not exhibit iron reduction in the absence of forced aeration. Adaptation of strain 41 to abrupt changes in oxygen input resulted in abruptly decreasing rates of Fe(II) and glucose consumption and probably in more "sparing" utilization of reductive equivalents. Such coordination of constructive and energy metabolism results probably from the fact that strain 41 is a natural mutant deprived of the ability to sporulate. However, all the sulfobacilli strains investigated exhibited a similar response to oxygen limitation (cultivation in a high layer of the medium under air partial pressure): growth rates and cell yields decreased, the rates and scale of iron oxidation and glucose consumption decreased, exometabolites were found in the medium, and part of the population entered the resting state. The latter is a characteristic response to oxygen deficiency: a specific autoregulatory system for synthesis of cell differentiation factors (d_1 factors) is induced, followed by transition to metabolic rest [27]. The physiological state of refractive forms was found to return to normal at enhanced oxygen diffusion. The respiration rates of the cells of moderately thermophilic *S. sibiricus* N1 grown under hindered oxygen diffusion and under intense aeration did not differ very significantly. This is an additional confirmation of adaptive capacity and viability of sulfobacilli under nonoptimal conditions, as well as of lability of their energy metabolism. For example, the respiration chain of strain N1 is branched and the stationary-phase cells experiencing deficiency of dissolved oxygen due to the high density of cell population utilize also the second pathway of electron transfer, with higher affinity to dissolved oxygen [28, 29]. Changes in the respiratory chains, synthesis of oxidases with high affinity to oxygen, and induction of alternative respiration systems under microaerobic conditions have been demonstrated for other organisms [30, 31]. Accumulation of exometabolites in the medium, characterization of the respiration rates of the cells grown without forced aeration, and the changes in cell morphology in thermoacidophilic sulfobacilli under such conditions, have not been previously reported. No exometabolites were detected in the medium under intense bubbling with air (1 volume of air per 1 volume of medium per min). Weak fermentation was also possible under oxygen limitation in the presence of glucose or its metabolites in the medium, as well as electron flow from yeast extract or its components (peptides, amino acids, nucleotides, etc.) to ferric iron with formation of acetate or propionate, or directly from these exometabolites to Fe(III). Sufficiently high respiration rates of the cells grown in a high level of the medium under air partial pressure may also indicate the possibility of rapid O_2 exhaustion by substrate-oxidizing cells and formation of microaerobic zones around them. Transition of the population to growth by substrate oxidation coupled to simultaneous Fe(III) reduction to Fe(II), together with transition of some vegetative cells to a resting state is a sound survival strategy [32]. The moderately thermophilic and thermotolerant strains *S. sibiricus* N1 and *S. thermotolerans* Kr1 employed this survival strategy under fixed O_2 content in the gas phase. For example, under hypoxia at 0.07% oxygen in the gas phase in the beginning and end of the experiment (practically anaerobic conditions), the cultures grew by oxidation of glycerol or yeast extract coupled to iron reduction. Under these conditions, cell yield

correlated with the content of ferrous iron (10 mM) and was close to the yield under organotrophic growth at intense aeration [14, 15]. The inability of these cultures to grow lithoautotrophically by tetrathionate oxidation under microaerobic conditions is probably due to the fact that growth on tetrathionate depends on the presence of exogenous yeast extract [14, 15] or on the changes in the cell membrane required for tetrathionate transport having not occurred [33].

Summarizing our experimental results, it can be stated that, under oxygen limitation, all the strains of thermophilic sulfobacilli used in this work are able to adapt to drastically changed conditions and to develop the best survival strategy. Study of the resting forms in sulfobacilli (alternative to spores) for their detailed characterization requires an independent experimental setup, as well as detailed investigation of the ability of these bacteria to switch between respiratory and fermentative metabolism.

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