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EXPERIMENTAL ARTICLES

Regulation of Metabolic Pathways in Sulfobacilli under Different Aeration Regimes

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Abstract—A comparative study of the activities of the enzymes of carbon metabolism from the cells of moderately thermophilic chemolithotrophic bacteria *Sulfobacillus sibiricus* (strains N1 and SSO) and *Sulfobacillus thermosulfidooxidans* subsp. *asporogenes* (strain 41) was carried out grown in a high layer of medium without forced aeration and cells grown with intense aeration. Limited air access to the growing *S. sibiricus* N1 cells resulted in switching from the pentose phosphate pathway of glucose metabolism to the Entner–Doudoroff pathway while the Embden–Meyerhof–Parnas pathway persisted. Irrespective of the level of the aeration, in the cells of *S. sibiricus* SSO and *S. thermosulfidooxidans* subsp. *asporogenes* 41, degradation of the glucose occurred via the Entner–Doudoroff and pentose phosphate metabolic pathways, respectively, as well as via the Embden–Meyerhof–Parnas pathway. Prolonged growth of *S. sibiricus*, strains N1 and SSO, in a high layer of the medium without forced aeration led to the repression of synthesis of most of the tricarboxylic acid cycle (TCA cycle) enzymes, in particular dehydrogenases, as well as of some carboxylases including RuBisCO. The traits of carbon metabolism in various strains of *Sulfobacillus* under conditions of oxygen deficiency are discussed.

Key words: acidothermophilic sulfobacilli, aeration conditions, carbon metabolism enzymes, TCA cycle, carboxylases.

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To survive in the changing environmental conditions, microorganisms need either to change the environment or to adapt themselves to its changes. Adaptation of an organism to the permanently varying conditions is a complicated process involving changes both at the genotype and phenotype levels. At the phenotypic level, the result of such adaptation is usually expressed as alterations in the metabolic processes and types of nutrition. Physiological and biochemical studies of numerous representatives of colorless sulfur-oxidizing bacteria resulted in a concept of highly diverse metabolic capacities among various taxonomic and physiological groups of these bacteria, among the microorganisms within such groups, and at the level of individual species and strains [1–3].

Thermophilic chemolithotrophic sulfur- and iron-oxidizing bacteria that belong to the genus *Sulfobacillus* represent one of the physiological groups existing in a constantly changing environment [4]. The habitats of these bacteria include exploited sulfide ore deposits, sites of thermogenesis, and reactors for bioleaching of sulfide ores and concentrates. In these habitats the bacteria are exposed to stresses, one of which is permanently varying oxygen concentration; the solubility of oxygen dramatically decreases with increasing temperature, in particular in acidic media enriched with the metal ions. At the same time, sulfobacilli are known to be facultative anaerobes and, therefore, to be able to grow in the absence of oxygen or at its low concentrations [5–7]. We showed earlier that sulfobacilli, which possess a wide range of the carbon metabolism enzymes, may rapidly reorganize their metabolism in response to the variations in nutrition conditions and physicochemical factors (high concentrations of metal ions, high and low temperature, extremely low pH values, etc.) [8–12].

The rate of the reorganization of the metabolic pathways is very important for the adaptation of an organism to environmental changes. However, no data describing the metabolic reorganization in sulfobacilli under the oxygen deficiency have been published.

In our previous study, we demonstrated that, under conditions of oxygen deficiency, when the cells were cultivated in a high medium layer with partial air pressure, the following changes were observed: the time of generation and growth duration increased, the cell yield decreased, exometabolites (acetate and propi-

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onate) were detected in the medium, dormant cells were formed, and the phase of iron oxidation was followed by the phase of iron reduction. Under conditions of hypoxia (0.07% oxygen in the gas phase), oxidation of the substrates was coupled with reduction of ferric iron as the terminal electron acceptor [13].

The goal of this work was to elucidate the effect of the level of aeration of the medium on the activity of the enzymes of carbon metabolism in chemolithotrophic moderately thermophilic bacteria *Sulfobacillus sibiricus* and *S. thermosulfidooxidans* subsp. *asporogenes*.

MATERIALS AND METHODS

Bacterial strains. Three moderately thermophilic strains of acidophilic bacteria of the genus *Sulfobacillus* were used throughout the study: *S. sibiricus*, strain $N1^T$ (VKM $B\text{-}2380^T$ = DSM 17363^T) and strain SSO; *S. thermosulfidooxidans* subsp. *asporogenes*, strain 41 (Institute of Microbiology, Armenia, $B\text{-}6981^T$). The strains were isolated from various habitats [11, 14–16].

Cultivation conditions. The strains N1, SSO, and 41 were cultivated under mixotrophic conditions in the media described earlier and containing Fe(II), $S_2O_3^{2-}$, glucose, and yeast extract [13]. The cells were grown at 55°C, pH 1.6–1.8. The cultures were inoculated with the cells grown on a rotary shaker at 180 rpm in 250-ml flasks with 100 ml of the medium. The V_{flask} : V_{medium} ratio was 2.5 : 1. In a set of experiments with *S. sibiricus* N1 at air partial pressure (21% oxygen in the gas phase), the strain was grown for ten transfers under static conditions in a high layer of the medium with ferrous iron (the V_{flask} : V_{medium} was 1.4 : 1).

Further cultivation of bacteria was conducted without forced aeration at air partial pressure in the bottles with $V_{bottle} : V_{medium} = 1.2–1.4 : 1$. Conditions of intense aeration achieved by bubbling of one volume of air through one volume of the medium per 1 min in 5-l bottles ($V_{bottle} : V_{medium} = 1.7 : 1$) were used as the control.

Analytical techniques. To determine the activity of the enzymes of carbon metabolism, the cells suspensions were prepared by centrifugation at 10000 *g*, 4°C, for 40 min [12]. The pellets were washed with acidified culture medium without energy sources. Then the cells were resuspended in 0.1 N Tris–HCl buffer, pH 6.8, and sonicated in a UZDN-2T sonicator for 3 min with 5- to 6-min intervals for cooling. The obtained suspension was centrifuged at 40000 *g*, 4°C, for 30 min. The activities of carboxylases, of the enzymes of the tricarboxylic acid cycle (TCA cycle), and of carbohydrate metabolism enzymes were measured in the supernatant using a Hitachi 200-20 spectrophotometer (Japan) and by the radiometric method [8]. The concentration of proteins was determined according to Lowry et al. [17].

The data, including the results of analytical determinations, were obtained in from three to five repetitions. The values of $p < 0.05$ were considered statistically significant.

RESULTS

The carbohydrate metabolism enzymes. Cultivation of *S. sibiricus* N1 at an air partial oxygen pressure under mixotrophic conditions in the medium with iron affected significantly the pathways of glucose metabolism (Table 1). Under these conditions, even after the first transfer activity of hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphofructokinase, 6-phosphogluconate dehydrogenase, 3-phosphoglyceroaldehyde dehydrogenase, and pyruvate kinase were significantly lower than in bacteria grown with intense aeration. The activity of the key enzymes of the Entner–Doudoroff pathway (6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase) was high. Further cultivation of strain N1 under the conditions of oxygen limitation (eleventh transfer) caused a decrease in the activity of most enzymes, including fructose bisphosphate aldolase.

These data suggested that, under optimal growth conditions (i.e., intense aeration), bacteria *S. sibiricus* N1 utilized glucose via the oxidative pentose phosphate and the Embden–Meyerhof–Parnas pathways [10]. If oxygen access to the growing culture oxygen supply is limited, carbohydrate metabolism switches over from the pentose phosphate to the Entner–Doudoroff pathway (Table 1).

The maximal activity of carbohydrate metabolism enzymes in *S. sibiricus* strain SSO was also detected in the cells grown under forced aeration. The enzymatic activities suggested that carbohydrate metabolism of this strain under intense aeration operates via the fructose bisphosphate pathway and the Entner–Doudoroff pathway (Table 1). In the absence of forced aeration, at limited oxygen diffusion, strain SSO employed the same metabolic pathways as strain N1, the fructose bisphosphate and the Entner–Doudoroff pathways. Under these conditions the activities of all enzymes in strain SSO decreased. Thus, notwithstanding the level of aeration, the pathways of glucose metabolism in strain SSO did not change.

In *S. thermosulfidooxidans* subsp. *asporogenes* 41 cultivated under conditions of atmospheric partial air pressure ($V_{bottle} : V_{medium} = 1.25 : 1$) in the medium containing ferrous iron and glucose, this carbohydrate is probably utilized via the glycolytic and the pentose phosphate pathways (Table 1). This suggestion is supported by the values of activities of the corresponding key enzymes involved in the Embden–Meyerhof–Parnas pathway, namely hexokinase, 6-phosphofructokinase, fructose-1,6-bisphosphate aldolase, triose phosphate dehydrogenase, and pyruvate kinase. The activity of the key enzyme of the pentose phosphate pathway, 6-phosphogluconate dehydrogenase, was five times higher than in the cells grown under conditions of intense aeration. Since the activities of 6-phospho-

Table 1. Activity of the enzymes of carbohydrate metabolism (nmol/(min mg protein)) in the cells of sulfobacilli grown under different regimes of aeration

Enzyme	*S. sibiricus* N1			*S. sibiricus* SSO		*S. thermosulfidooxidans* subs. *asporogenes*	
	Intense aeration	Without intense aeration (first transfer)	Without intense aeration (11th transfer)	Intense aeration	Without intense aeration	Intense aeration	Without intense aeration
Hexokinase, EC 2.7.1.1	42.4	13.6	3.3	8.3	2.7	28.5	39.2
Glucose-6-phosphate dehydrogenase, EC 1.1.1.49	215.0	64.4	28.8	7.8	2.0	101.2	40.0
6-Phosphogluconate dehydrogenase, EC 1.1.1.43	12.1	3.7	ND	ND	ND	4.2	22.3
6-Phosphogluconate dehydratase, EC 4.2.1.12 + 2-keto-3-deoxy-6-phosphogluconate aldolase, EC 4.1.2.14	ND	36.2	11.4	23.3	8.9	0.9	ND
Fructose-1,6-bisphosphate aldolase, EC 4.1.2.13	424.5	336.0	198.8	350.0	256.0	640.0	1175.0
6-Phosphofructokinase, EC 2.7.1.11	46.5	9.0	8.1	11.7	7.2	4.5	19.0
3-phosphoglycerol aldehyde dehydrogenase, EC 1.2.1.12	316.3	38.7	19.2	20.6	14.5	94.6	105.9
Pyruvate kinase, EC 2.7.1.40	81.8	8.0	8.9	8.3	4.9	10.9	9.8

Note: In this and other tables, "ND" indicates that the activities of these enzymes were not detected under the experimental conditions.

gluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase were not detected, the Entner–Doudoroff pathway was probably not functional under oxygen deficiency conditions. However, in the cells grown under intense aeration the activities of those enzymes were shown to remain at a low level. Thus, cultivation of the asporogenous strain in the absence of intense aeration led to increased activities of half of the investigated enzymes of carbohydrate catabolism compared to those measured in the cells grown under normoxia conditions (Table 1).

Carboxylases. The cells of *S. sibiricus* N1 grown mixotrophically under atmospheric partial air pressure contained the key enzyme of the Calvin cycle, ribulosebisphosphate carboxylase/oxygenase (RuBisCO/O). However, the activity of this enzyme was only 2.2 nmol of CO_2/(min mg protein) (Table 2). In the cell extracts obtained after repeated transfers in the same conditions (11 transfers), the activity of RuBisCO decreased tenfold. At the same time, in *S. sibiricus* N1 cells cultivated under mixotrophic conditions with intense aeration in the medium with iron, the activity of RuBisCO achieved 7.0 nmol of CO_2/(min mg protein). The activities of pyruvate carboxylase, phosphoenolpyruvate carboxylase (PEP carboxylase), and PEP carboxytransphosphorylase in extracts of the cells grown under oxygen deficiency, especially after numerous transfers under the same conditions, were low or not detected. Their activities were many times lower than the activities of the corresponding carboxylases in the extracts of the cells grown under intense aeration (Table 2).

The *S. sibiricus* SSO cells contain RuBisCO, the key enzyme of the Calvin cycle, RuBisCOexhibiting activity of 3.5 and 1.7 nmol of CO_2/(min mg protein) at the control and experimental oxygen regimes, respectively (Table 2). PEP carboxylase, PEP carboxykinase, and PEP carboxytransphosphorylase were also detected in the cells of strain SSO grown under the above conditions. Pyruvate kinase was found only in the cells of the SSO strain grown under normoxia conditions, although at a low level.

When *S. thermosulfidooxidans* subsp. *asporogenes* 41 was cultivated without forced aeration in bottles ($V_{bottle} : V_{medium} = 1.25 : 1$) in the presence of ferrous iron and 2.75 mM glucose, the RuBisCO/O activity was found to be lower (7.5 nmol of CO_2/(min mg protein)) than in the cells grown under intense aeration (Table 2). Limited diffusion of oxygen to the cells resulted in a 20-fold decrease of PEP carboxylase activity (1.0 nmol CO_2/(min mg protein)) compared to the activity of the enzymes in the cells grown without oxygen limitation. Pyruvate carboxylase activity (0.3 nmol of CO_2/(min mg protein)) did not depend on the rate of oxygen diffusion. In strain 41 grown under air partial oxygen pressure, activities of the car-

Table 2. Activity of the carboxylation enzymes (nmol CO_2/(min mg protein)) in the cells of sulfobacilli grown under different oxygen regimes

Enzyme	*S. sibiricus* N1			*S. sibiricus* SSO		*S. thermosulfidooxidans* subsp. *asporogenes* 41	
	Intense aeration	Without intense aeration (first transfer)	Without intense aeration (11th transfer)	Intense aeration	Without intense aeration	Intense aeration	Without intense aeration
Ribulose bisphosphate carboxylase/oxygenase, EC 4.1.1.39	7.0	2.2	0.2	3.5	1.7	18.0	7.5
Pyruvate carboxylase, EC 6.4.1.1	9.8	0.3	0.1	0.4	ND	0.3	0.3
PEP carboxylase, EC 4.1.1.31	0.6	0.2	0.1	0.7	0.4	20.7	1.0
PEP carboxykinase, EC 4.1.1.49	ND	ND	ND	1.3	0.3	ND	1.2
PEP carboxytransphosphorylase, EC 4.1.1.38	0.7	0.3	ND	0.9	0.2	ND	0.7

Table 3. Activity of the TCA cycle enzymes (nmol/(min mg protein)) in the cells of sulfobacilli grown under different oxygen regimes

Enzyme	*S. sibiricus* N1			*S. sibiricus* SSO	
	Intense aeration	Without intense aeration (first transfer)	Without intense aeration (11th transfer)	Intense aeration	Without intense aeration
Citrate synthase, EC 4.1.3.7	13.6	7.2	6.1	22.3	17.5
Aconitate hydratase, EC 4.2.1.3	26.6	20.6	31.5	13.9	7.3
Isocitrate dehydrogenase, EC 1.1.1.42	197.3	127.1	8.4	52.5	15.6
2-Oxoglutarate dehydrogenase, EC 1.2.4.2	ND	ND	ND	ND	ND
Succinate dehydrogenase, EC 1.3.99.1	38.9	27.3	14.4	20.0	8.7
Fumarate hydratase, EC 4.2.1.2	66.8	68.4	54.2	151.7	25.2
Malate dehydrogenase, EC 1.1.1.37	490.1	472.2	37.5	33.4	17.1
Malate synthase, EC 4.1.3.2	10.5	–	–	10.2	–

boxylases responsible for heterotrophic CO_2 assimilation (PEP carboxytransphorylase and PEP carboxykinase) were 0.7 and 1.2 nmol of CO_2/(min mg protein), respectively. No activity of these enzymes was detected in the cells cultivated with intense aeration.

The TCA cycle enzymes. In *S. sibiricus* N1 and SSO, the activities of the enzymes catalyzing the terminal stages of oxidation of organic substances (the TCA cycle enzymes) were determined. Cultivation of *S. sibiricus* N1 in a high layer of medium under oxygen limitation did not affect the activity of aconitate hydratase and fumarate hydratase (Table 3). After repeated transfers under these conditions, the activity of the enzymes was the same as in bacteria grown under optimal aeration. Nevertheless, activity of citrate synthase, the key enzyme of the TCA cycle was two times lower in the absence of intense aeration. Furthermore, already at the first passage of strain N1, when it was transferred from the control conditions to the oxygen deficient conditions, the activity of all the dehydrogenases decreased somewhat. Further cultivation under limited oxygen access caused a more pronounced effect on the activity of hydrogenases: activity of the enzymes was two to ten times less (Table 3).

Due to the absence of 2-oxoglutarate dehydrogenase activity, the complete TCA cycle is not functional in the cells of *S. sibiricus* SSO and *S. sibiricus* N1, irrespective of the level of aeration. One of the enzymes of the glyoxylate cycle, malate synthase, was determined in the control SSO culture (intense aeration); however, another one, isocitrate lyase, was not detected. It was not revealed in other sulfobacilli either [8–10, 12].

The extent of aeration did not affect significantly the activity of citrate synthase in strain SSO. On the contrary, oxygen limitation resulted in a sixfold and twofold decrease in activity of fumarate hydratase and aconitate hydratase, respectively. Synthesis of dehydrogenases was also inhibited, and their activity decreased two- to threefold (Table 3).

Thus, in both *S. sibiricus* strains, N1 and SSO, the biosynthesis of dehydrogenases of the TCA cycle was the most sensitive to decreased content of oxygen, the main electron acceptor in the medium. Activities of other enzymes involved in the TCA cycle responded to drastic changes in aeration intensity in the opposite way. Although prolonged exposure to the stress factor caused more pronounced repression of dehydrogenases synthesis, complete repression of the TCA cycle enzymes was not observed.

DISCUSSION

Analysis of the presence and activity of the enzymes of carbon metabolism in the cells of moderately thermophilic strains *S. sibiricus* N1 and SSO and *S. thermosulfidooxidans* subsp. *asporogenes* 41 was carried out. Such analysis has never been carried out by other authors who conducted similar studies. Our study allowed us to conclude that after abrupt changes in the conditions of oxygen supply (from intense aeration to the limited oxygen diffusion), bacteria were able to grow using the substrate-level (glucose utilization; oxidation of sulfite involving APS reductase; see below) and oxidative phosphorylation. The cells grown under oxygen limitation reduced iron and demonstrated high respiration rates when transferred to a fresh medium [13].

The studied bacteria in general were found to have flexible carbon metabolism. Strains N1 and 41 exhibited the most labile carbohydrate metabolism. The prevailing pathway of glucose metabolism in these cultures depended on the aeration level.

Prolonged growth of *S. sibiricus* N1 at decreased oxygen diffusion rate caused repression of most enzymes involved in the TCA cycle, especially of dehydrogenases. Carboxylases, including RuBisCO, the key enzyme of Calvin cycle, were repressed as well. This decrease of activity of the enzymes probably resulted in part from the metabolic dormancy of refractory cells formed under the conditions of oxygen deficiency [13, 18]. The analyzed strains differed significantly from each other in the activities of carbon metabolism enzymes. Under normoxia conditions, the activities of dehydrogenases participating in carbohydrate metabolism and supplying the reducing equivalents were 15–20 times higher in strain N1 than in strain SSO. Such differences could be explained by the differences in the natural habitats of the strains. These strains were isolated from sulfide ores with different contents of Fe(II), Fe(III), heavy metal ions, H^+ concentrations, aeration and temperatures [14, 15]. In the processes of bioleaching (the habitat of N1 strain), the most active organisms stable to the extreme conditions survive.

The activities of dehydrogenases, kinases, some carboxylases of $^{14}CO_2$ heterotrophic fixation and fructose-1,6-bisphosphate aldolase increased in the natural mutant, asporogenous strain 41, at cultivation in a high layer of the medium. Since they preserve the activity of the vitally important enzymes, such organisms, which are not able to sporulate, have some advantages in the environment and may acquire survival advantages during heap leaching of sulfide minerals under oxygen limitation [16].

Thus, transition from aerobic cultivation of acidothermophilic bacteria to growth under oxygen deficiency conditions resulted in essential reorganization of carbon metabolism and in changes in the enzymatic activity of certain metabolic pathways. Oxygen played also the regulatory role in selection of the pathway of energy metabolism [13].

Evolution of acidophilic chemolithotrophic bacteria of the genus *Sulfobacillus* probably led in the direction of development of mixotrophic metabolism. This type of metabolism implies simultaneous utilization of organic and inorganic compounds as energy and carbon sources at high temperature and low content of oxygen and carbon dioxide (the solubility of gases decreases with an increase in temperature), especially in acidic media enriched with metal ions. Such extreme, permanently changing environment required more complex maintenance systems, more complicated genome, and consequently more complex enzymatic apparatus providing sulfobacilli with more extensive metabolic capacities (exceeding those of obligate aerobes) under various aeration conditions.

As a result, similar to *Acidithiobacillus ferrooxidans* [19] sulfobacilli obtained the ability to switch their metabolism between alternative pathways and to move to anaerobiosis. As facultative Fe(III) reducers, they can participate in the final stages of organic matter dissimilation during mixotrophic growth under anaerobic conditions. Reduction of Fe(III) in this case occurs according to the following equation: $Fe(OH)_3 + 3H^+ + e^- \longrightarrow Fe^{2+} + 3H_2O$. Under oxygen deficiency, sulfobacilli also preserve their ability to oxidize sulfur and sulfite (including colloidal sulfur and sulfite resulting from the chemical transformation of thiosulfate [20]) using the enzymes described in our works for the first time. These enzymes coupling the processes of oxidation of inorganic reduced sulfur compounds and Fe(III) reduction include sulfur : Fe^{3+} oxidoreductase and sulfite : Fe^{3+} oxidoreductase: $S^0 + 4Fe^{3+} + 3H_2O \longrightarrow H_2SO_3 + 4Fe^{2+} + 4H^+$ and further $H_2SO_3 + 2Fe^{3+} + H_2O \longrightarrow H_2SO_4 + 2Fe^{2+} + 2H^+$ [7].

Thus, *S. sibiricus*, strains N1 and SSO, can oxidize sulfur to sulfite via sulfur dioxygenase under aerobic conditions or via sulfur : Fe^{3+} oxidoreductase under oxygen limitation. Sulfite is further oxidized to sulfate in reactions catalyzed by sulfite oxidase, APS reductase (adenosine phosphosulfate reductase) and sulfite : Fe^{3+} oxidoreductase. Oxidation of sulfur and sulfite by sulfur : Fe^{3+} oxidoreductase, sulfur dioxygenase (?), sulfite oxidase, and sulfite : Fe^{3+} oxidoreductase yields energy in the electron transport chain, while action of APS reductase is related to substrate phosphoryla-

tion. We demonstrated the activities of sulfur and sulfite : Fe^{3+} oxidoreductases in *S. sibiricus* strains; these enzymes were constitutive. The study of the presence of similar oxidoreductases in other strains of sulfobacilli grown both under oxygen deficiency and intense aeration conditions will be continued.

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