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EXPERIMENTAL ARTICLES

Activity of the Enzymes of Carbon Metabolism in *Sulfobacillus sibiricus* under Various Conditions of Cultivation

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Abstract—The thermoacidophilic iron-oxidizing chemolithotroph *Sulfobacillus sibiricus* $N1^T$ is characterized by steady growth and amplified cell yield when grown in vigorously aerated medium containing Fe^{2+}, glucose, and yeast extract as energy sources. In this case, carbon dioxide, glucose, and yeast extract are used as carbon sources. Glucose is assimilated through the fructose-bisphosphate pathway and the pentose-phosphate pathway. The glyoxylate bypass does not function in *S. sibiricus*, and the tricarboxylic acid cycle is disrupted at the level of 2-oxoglutarate dehydrogenase. The presence of ribulose-bisphosphate carboxylase indicates that carbon dioxide fixation proceeds through the Calvin cycle. The activity of ribulose-bisphosphate carboxylase is highest in autotrophically grown cells. The cells also contain pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate carboxytransphosphorylase.

Key words: Sulfobacillus sibiricus, growth, enzymes of carbon metabolism, tricarboxylic acid cycle (TCA cycle), carboxylases.

Aerobic moderately thermophilic chemolithotrophic spore-forming bacteria of the genus *Sulfobacillus*, which oxidize Fe^{2+}, S^{2-}/S^0 compounds, and sulfide minerals, have an extremely flexible metabolism, exhibiting features of both autotrophy and heterotrophy. Mixotrophic conditions are most preferable for sulfobacilli: these microorganisms can generate energy by simultaneous oxidation of organic substances (yeast extract, some amino acids, glucose, etc.) and inorganic compounds (e.g., reduced iron and sulfur compounds and metal sulfides) and can simultaneously use carbon dioxide and organic substrates as carbon sources.

By now, carbon metabolism has been studied in *Sulfobacillus thermosulfidooxidans* [1–3] and *S. acidophilus* [4, 5]. In *S. thermosulfidooxidans*, the utilization of organic substrates may proceed through the fructose-bisphosphate pathway, the pentose-phosphate pathway, and the Entner–Doudoroff pathway with the participation of certain anaplerotic reactions of carboxylation. In *S. acidophilus* ALV, carbohydrate degradation proceeds mainly through the pentose-phosphate pathway. In certain *S. thermosulfidooxidans* strains (41, 1269, K1), energy and carbon sources determine the primary carbon metabolism pathway [1, 3, 6]. Such a flexibility of metabolism may help these microorganisms withstand stresses and substrate limitation.

While testing a technology of bioleaching of gold-arsenic concentrates, a novel aerobic gram-positive spore-forming thermoacidophilic bacterium was isolated. This microorganism is a member of the association of mesophilic and moderately thermophilic bacteria which oxidize sulfide minerals of the pulp in the reactor in the process of bioleaching at 30°C [7]. On the basis of genetic and physiological characteristics, it was classified as *S. sibiricus* sp. nov. [7].

This paper presents the results of our study of the carbon metabolism in *S. sibiricus* grown under intense aeration at an optimal temperature of 55°C in a medium containing Fe^{2+}, glucose, and yeast extract.

MATERIALS AND METHODS

Sulfobacillus sibiricus $N1^T$ (VKM B-2280, All-Russia Collection of Microorganisms) was used throughout this study. The strain was isolated from the ore concentrate from Nezhdaninskoe deposit (Eastern Siberia, Sakha) and maintained in modified 9K medium [8] containing (g/l distilled water) $(NH_4)_2SO_4$, 3.0; KCl, 0.1; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $Ca(NO_3)_2$, 0.01; yeast extract, 0.2; $FeSO_4 \cdot 7H_2$, 35 mM. The initial pH was adjusted to 1.8–1.9 with 10 N H_2SO_4. The inoculum was added to a fresh medium in the amount of 5–10 vol % and cultivated at 55°C. For mixotrophic conditions, 1 mM glucose was added to the medium. The efficacy of the autotrophic growth of the strain was determined in a mineral medium containing Fe^{2+} as a single source of electrons; for the determination of the efficacy of organotrophic growth, a medium containing 1 mM glucose and 0.2 g/l yeast extract and no mineral

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Table 1. Activity of the enzymes of carbon metabolism (nmol/(min mg protein)) in *S. sibiricus* N1 cells grown under various conditions

Enzyme	Conditions of growth		
	autotrophic	heterotrophic	mixotrophic
Hexokinase	20.7	26.7	42.4
Glucose-6-phosphate dehydrogenase	184.7	312.7	315.0
6-Phosphogluconate dehydrogenase	6.3	26.9	12.1
6-Phosphogluconate dehydratase + 2-keto-3-deoxy-6-phosphogluconate aldolase	n.d.	n.d.	n.d.
Fructose bisphosphate aldolase	585.2	542.3	424.5
Phosphofructokinase	16.2	61.5	46.5
Glyceraldehyde-3-phosphate dehydrogenase	238.8	423.1	316.3
Pyruvate kinase	25.6	75.1	81.8

Note: In this and other tables, "n.d." means that the activity was not detected under the conditions of the experiment.

sources of energy was used. The inoculum was grown in 250-ml flasks containing 100 ml of medium with shaking (180 rpm) at 55°C. Then the cells were transferred to a 5-l flask containing 3 l of the medium. Intense aeration was performed by passing one volume of air through one volume of medium per min. Fe^{3+}, Fe^{2+}, and glucose quantities were determined with the use of methods described earlier [1, 3]. Culture growth was monitored by counting cells in a Goryaev–Tom chamber. The products of metabolism of organic substrates were analyzed using the methods of gas-liquid chromatography on a Gaz-Khrom 3700 chromatograph (Sovpol column with a length of 1.5 m; temperature, 190°C; argon as the carrier gas; flame-ionization detector).

Enzymatic activity was determined in cells in the late exponential phase of growth. The cells were harvested by centrifugation at 10000 *g*, washed with a 0.05 M Tris–HCl buffer, pH 7.4, resuspended in the same buffer, and subjected to sonication five times for 1 min. At the end of sonication, the homogenate was centrifuged at 40000 *g* for 20 min, and the supernatant was assayed for enzymatic activities with the use of spectrophotometric methods.

Hexokinase activity (EC 2.7.1.1) was determined from NADP reduction in the presence of glucose, ATP, and glucose-6-phosphate dehydrogenase [9]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activities were determined from NADP reduction in the presence of glucose-6-phosphate or 6-phosphogluconate, respectively [9]. 6-Phosphogluconate dehydratase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate (3-deoxy-2-oxo-6-phosphogluconate) aldolase (EC 4.1.2.14) activities were determined simultaneously from NADH oxidation in the presence of 6-phosphogluconate and lactate dehydrogenase [10]. Phosphofructokinase (EC 2.7.1.11) activity was judged from NADH oxidation in the presence of fructose-6-phosphate [11]. Fructose-bisphosphate aldolase (EC 4.1.2.13) activity was judged from triose quantities generated from fructose bisphosphate [12]. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activity was determined from NADH oxidation in the presence of 3-phosphoglycerate [9]. Pyruvate kinase (EC 2.7.1.40) activity was judged from NADH oxidation in the presence of phosphoenolpyruvate and lactate dehydrogenase [9].

Citrate synthase (EC 4.1.3.7) activity was assessed by monitoring at 412 nm the generation of CoA-SH in the reaction with 5,5'-dithiobis-(2-nitrobenzoate) [13]. Aconitase (EC 4.2.1.3) activity was assessed by monitoring the increase in the optical density at 240 nm in the presence of isocitrate [14]. Isocitrate dehydrogenase (EC 1.1.1.4) activity was determined from NADP reduction in the presence of isocitrate [9]; 2-oxoglutarate dehydrogenase (EC 1.2.4.2) activity was judged from NAD reduction in the presence of 2-oxoglutarate, thiamine pyrophosphate, and coenzyme-A [15]. Succinate dehydrogenase (EC 1.3.99.1) activity was determined from ferricyanide reduction monitored at 420 nm [16]. Fumarase (EC 4.2.1.2) activity was assessed by monitoring the decrease in the optical density at 290 nm in the presence of fumarate [9]. Malate dehydrogenase (EC 1.1.1.37) activity was judged from NADH oxidation in the presence of oxaloacetic acid [9]. Isocitrate lyase (EC 4.1.3.1) activity was monitored in the reaction of glyoxylate generation from isocitrate [17]. Malate synthase (EC 4.1.3.2) activity was measured by monitoring the increase in the optical density at 412 nm in the presence of 5,5'-dithiobis-(2-nitrobenzoate) [18]. All the measurements were conducted at room temperature, except for the aldolase activity. Enzymatic activities were measured on a Hitachi 200-20 spectrophotometer. Carboxylase activities were assessed radiometrically [19]. Protein content was determined as described earlier [1].

RESULTS AND DISCUSSION

The growth of *S. sibiricus* under intense aeration. The phenotypic characteristics of strain N1^T, representing the novel species *S. sibiricus*, were described recently [7]. It was found that two or three succeeding subcultures are able to grow in a medium containing Fe^{2+} as a single energy source, and eight, in a medium containing yeast extract as the energy and carbon source. The results of the present study confirm the data on the unstable growth of strain N1 in media containing only a mineral or only an organic source of energy, including a medium containing 1 mM glucose and 0.2 g/l yeast extract, which was not tested in previous studies [7].

The number of cells after the first cultivation in the Fe^{2+}-containing medium amounted to 8×10^7 per 1 ml. The generation time was 8 h. The cells oxidized up to 80% of the reduced iron during their growth (Fig. 1). In the medium for organotrophic growth containing yeast extract and 1 mM glucose, cells consumed up to 40% of glucose after 14 h of incubation. The minimal generation time was 3.5–4 h; the number of cells amounted to $1–3 \times 10^8$ per 1 ml. During 12 h of growth in a medium containing both mineral and organic sources of energy, cells oxidized 100% of Fe^{2+} and also consumed glucose (residual glucose concentration was 0.5 mM). No intermediates of carbon metabolism were detectable. Minimal generation time was 2 h; the number of cells amounted to 5×10^8 per ml.

In order to evaluate catabolic processes in *S. sibiricus* under various growth conditions, we studied the activity of key enzymes of its carbon metabolism.

Enzymes of carbon metabolism. In all of the tested media under aerobic conditions, the key enzymes of the pentose-phosphate pathway (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase) and of the Embden–Meyerhof–Parnas pathway (phosphofructokinase, fructose bisphosphate aldolase) were found in *S. sibiricus* (Table 1). 6-Phosphogluconate hydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase were not found, which indicates that in *S. sibiricus* sugar degradation does not proceed through the Entner–Doudoroff pathway, as distinct from the *S. thermosulfidooxidans* strains 1269 and 41 and the thermotolerant strain K1 [3].

The activity of hexokinase, glucose-6-phosphate dehydrogenase, and pyruvate kinase in *S. sibiricus* was highest after growth under mixotrophic conditions in the presence of glucose, which was also characteristic of other sulfobacilli studied by us. However, in contrast to other strains, the activity of 6-phosphogluconate dehydrogenase in strain N1 was higher under heterotrophic conditions than under mixotrophic conditions. So was the activity of phosphofructokinase, fructose bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase. This may indicate that the Embden–Meyerhof–Parnas pathway in *S. sibiricus* is of greater importance under heterotrophic conditions than under mixotrophic (optimal) conditions.

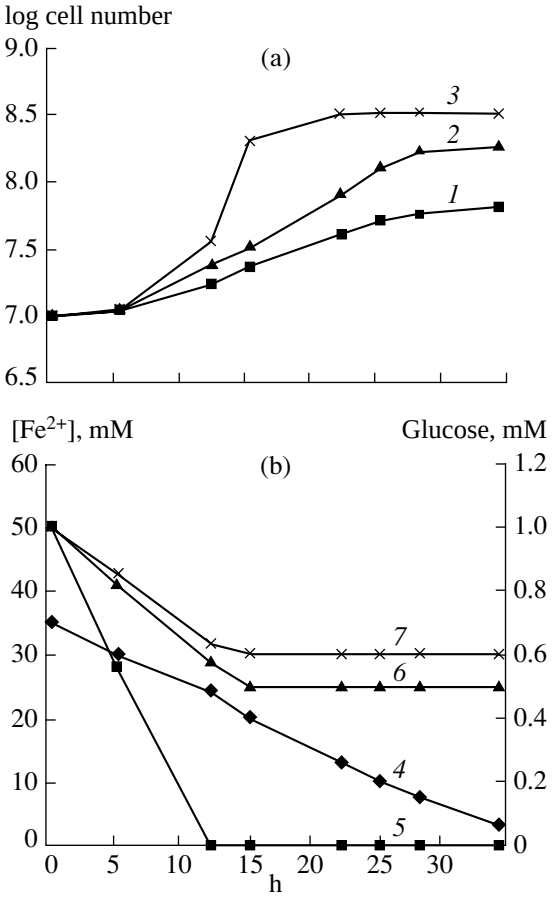

Fig. 1. (a) Growth of the moderately thermophilic acidophilic bacterium *Sulfobacillus sibiricus* N1 under (*1*) autotrophic, (*2*) heterotrophic, and (*3*) mixotrophic conditions and (b) glucose consumption under (*6*) mixotrophic and (*7*) heterotrophic conditions and iron oxidation under (*4*) autotrophic and (*5*) mixotrophic conditions.

The enzymes of the pentose-phosphate and Embden–Meyerhof–Parnas pathways were also found in autotrophically grown cells, but their activities were lower than those found in cells grown under heterotrophic or mixotrophic conditions, except for fructose bisphosphate aldolase. The activity of fructose bisphosphate aldolase was rather high, which indicates that it may be involved in polysaccharide synthesis.

The results indicate that in *S. sibiricus* grown under intense aeration, glucose degradation proceeds through the pentose-phosphate and Embden–Meyerhof–Parnas pathways.

Enzymes of the TCA cycle. In *S. sibiricus*, the terminal steps of organic substrate degradation proceed through certain reactions of the TCA cycle (Table 2). As in *S. thermosulfidooxidans* 1269 and 41, the TCA cycle cannot function as a whole due to the absence of 2-oxoglutarate dehydrogenase. Aconitase was also not

Table 2. Activity of the enzymes of the tricarboxylic acid cycle (nmol/(min mg protein)) in *S. sibiricus* N1 cells grown under various conditions

Enzyme	Conditions of growth		
	autotrophic	heterotrophic	mixotrophic
Citrate synthase	14.4	3.8	13.6
Aconitase	n.d.	n.d.	26.6
Isocitrate dehydrogenase	42.8	77.4	197.3
Succinate dehydrogenase	91.4	55.3	38.9
Fumarase	76.5	22.8	66.8
Malate dehydrogenase	300.3	653.8	490.1
Malate synthase	23.1	13.5	10.5

Table 3. Activity of carboxylation enzymes (nmol/(min mg protein)) in *S. sibiricus* N1 cells grown under various conditions

Enzyme	Conditions of growth		
	autotrophic	heterotrophic	mixotrophic
Ribulose-bisphosphate carboxylase (EC 4.1.1.39)	12.8	4.8	7.0
Pyruvate carboxylase (EC 6.4.1.1)	0.5	1.1	9.8
Phosphoenolpyruvate carboxylase (EC 4.1.1.31)	1.8	2.2	0.6
Phosphoenolpyruvate carboxykinase (EC 4.1.1.32)	4.1	n.d.	n.d.
Phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38)	0.4	n.d.	0.7

found in autotrophically and heterotrophically grown *S. sibiricus* cells, whereas its activity in mixotrophically grown cells was rather high, although much lower than in strain K1 [20].

The activity of citrate synthase, the key enzyme of the TCA cycle, was also rather high, as in other sulfobacilli studied. However, it dropped in the cells grown on organic medium. The activity of isocitrate dehydrogenase in strain N1 was highest under mixotrophic conditions, but the activities of succinate dehydrogenase and malate dehydrogenase were higher during organotrophic growth. This may indicate that in the medium containing only organic sources of energy, dehydrogenases supply electron transport chain with electrons, whereas under mixotrophic conditions organic substrates are primarily used as carbon sources and reduced iron compounds are used as the energy source.

S. sibiricus exhibited higher activity of malate synthase (a glyoxylate cycle enzyme) than other sulfobacilli studied. However, isocitrate lyase activity was not found in *S. sibiricus*, which indicates the absence of the glyoxylate cycle, as in other known microorganisms of this group [1, 2, 20].

Carboxylases. Ribulose bisphosphate carboxylase (RuBFC), the key enzyme of the Calvin cycle, was found in *S. sibiricus* cells grown in all of the media tested, including the medium containing only organic sources of energy, (an analogous pattern of RuBPC biosynthesis is characteristic of in *S. thermosulfidooxidans* [2]. The activity of RuBPC was highest in autotrophically grown cells (Table 3), where it was twice as high as in cells grown under mixotrophic conditions. In the medium containing only organic sources of energy, the activity of RuBPC amounted to 4.8 nmol/(min mg protein).

S. sibiricus also exhibited pyruvate carboxylase activity, which was highest in cells under mixotrophic conditions, and phosphoenolpyruvate carboxylase activity, which was rather low under all the conditions tested (Table 3). The activity of phosphoenolpyruvate carboxytransphosphorylase was also low, both under autotrophic and mixotrophic conditions (Table 3). Phosphoenolpyruvate carboxykinase activity was measured only in autotrophically grown cells (Table 3).

Summarizing the results, we conclude that *S. sibiricus*, like other known sulfobacilli, possesses efficient regulatory mechanisms that provide for the diversity of metabolic pathways.

As the data on the enzymatic activities show (Table 1), *S. sibiricus* is close to *S. acidophilus* ALV and the thermotolerant strain K1 grown heterotrophically [1, 3, 5]. Like in strain ALV but in contrast to strain K1, the Entner–Doudoroff pathway does not function in strain N1. In addition, *S. sibiricus* is characterized by higher activities of pyruvate kinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, which supply electron transport chain with reducing equivalents. This suggests that organic energy sources are used by *S. sibiricus* more efficiently than by *S. thermosulfidooxidans*.

The growth of *S. sibiricus* under heterotrophic conditions is unstable, as is the growth of some other moderately thermophilic sulfobacilli. However, the enzymatic activities (Table 1) and the amount of sugar utilized indicate that heterotrophic conditions are more favorable for *S. sibiricus* than for *S. thermosulfidooxidans* 1269 and 41 [1, 3].

As in other sulfobacilli studied, the complete TCA cycle and the glyoxylate cycle do not function in *S. sibiricus* N1. Carbon dioxide fixation proceeds through the Calvin cycle and via some other reactions of carboxylation.

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