Microbiology, Vol. 71, No. 6, 2002, pp. 651–656. Translated from Mikrobiologiya, Vol. 71, No. 6, 2002, pp. 755–761.

EXPERIMENTAL ARTICLES

The Enzymes of Carbon Metabolism in the Thermotolerant Bacillar Strain K1

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Received June 10, 2002

Abstract—To determine enzymatic activities in the thermotolerant strain K1 (formerly *"Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans"*), it was grown in a mineral medium with (1) thiosulfate and Fe^{2+} or pyrite (autotrophic conditions), (2) Fe^{2+}, thiosulfate, and yeast extract or glucose (mixotrophic conditions), and (3) yeast extract (heterotrophic conditions). Cells grown mixo-, hetero-, and autotrophically were found to contain enzymes of the tricarboxylic acid (TCA) cycle, as well as malate synthase, an enzyme of the glyoxylate cycle. Cells grown organotrophically in a medium with yeast extract exhibited the activity of the key enzymes of the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways. The increased content of carbon dioxide (up to 5 vol %) in the auto- and mixotrophic media enhanced the activity of the enzymes involved in the terminal reactions of the TCA cycle and the enzymes of the pentose phosphate pathway. Carbon dioxide is fixed in the Calvin cycle. The highest activity of ribulose bisphosphate carboxylase was detected in cells grown autotrophically at the atmospheric content of CO_2 in the air used for aeration of the growth medium. The activities of pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate carboxytransphosphorylase decreased with increasing content of CO_2 in the medium.

Key words: thermotolerant sulfobacilli, enzymes of carbohydrate metabolism, tricarboxylic acid cycle, carboxylases.

Our previous studies made it possible to reveal some genotypic, physiological, and biochemical differences of the thermotolerant strain K1 from the moderately thermophilic *Sulfobacillus thermosulfidooxidans* strains 1269^T and 41 [1–3]. Strain 41 is a natural asporogenic mutant of *S. thermosulfidooxidans.* Strain K1 was described earlier as a thermotolerant subspecies of this species [4]. The 16S rRNA sequence analysis of strain K1 showed its 87.7% genetic similarity to the type strain *S. thermosulfidooxidans* 1269^T. DNA–DNA reassociation data indicate a low level of DNA homology (31–37%) between strain K1 on the one hand and strains 41 and 1269^T on the other hand. The pulsed-field gel electrophoresis of chromosomal DNA from strain K1 also showed that this strain differs from other *S. thermosulfidooxidans* strains. In physiological properties, strain K1 is closer to *Sulfobacillus disulfidooxidans* and to alicyclobacilli than to other *S. thermosulfidooxidans* strains.

Upon organotrophic growth, strain K1 is more stable than strains 1269^T and 41 and is characterized by an increased ability to utilize glucose as the source of energy, as well as higher values of the growth rate, biomass yield, and activities of some enzymes of carbohydrate metabolism [3]. During the autotrophic growth of strain K1 in a medium with Fe^{2+} and thiosulfate, its growth parameters (growth rate and biomass yield) and enzymatic activities are lower than those of moderate thermophiles of the species *S. thermosulfidooxidans* [3].

The degree of utilization of inorganic electron donors (Fe^{2+}, S^0, and sulfide minerals) under mixotrophic conditions differs in various sulfobacilli: some of them actively and almost completely oxidize Fe^{2+}, S^0, and FeS_2 but slowly oxidize organic substrates [3, 5, 6], whereas others (such as strain K1 and *S. disulfidooxidans* strain SD-11) nearly completely oxidize organic substances but only slightly inorganic substrates (Fe^{2+} by no more than 20%) [3, 7]. The study of metabolic pathways in sulfobacilli should provide better insight into their genotypic and taxonomic peculiarities.

In this work, we continued the study of the enzymes of carbohydrate metabolism in the thermotolerant strain K1 in order to gain a better understanding of the terminal stages of the oxidation of organic substances and biosynthetic reactions in this strain. Strains 1269^T and 41 grown mixo-, hetero-, and autotrophically contain enzymes of the Calvin cycle and some anaplerotic carboxylases, whose activity depends on growth conditions. The tricarboxylic acid (TCA) cycle in these strains cannot operate because of the absence of 2-oxoglutarate dehydrogenase and of the glyoxylate cycle. The low activities of the enzymes of carbohydrate

0026-2617/02/7106-0651$27.00

metabolism and the TCA cycle under hetero- and autotrophic conditions explain, to a certain degree, the reasons for the unstable growth of moderately thermophilic sulfobacilli under such conditions [8, 9]. Raising the concentration of CO_2 in the medium stabilizes the activity of enzymes of the TCA cycle in the autotrophically grown strains 1269^T and 41.

Presently, it is difficult to compare the thermotolerant strain K1 and other sulfobacilli on the basis of data on the maximal biomass yield and doubling time during organotrophic growth in a medium with yeast extract as the sole source of energy (it is these data that were used by Norris *et al.* [6] to classify sulfobacilli). However, the study of the growth parameters and relevant enzymatic activities should allow strain K1 to be ascribed either to the *S. thermosulfidooxidans* group or to the *S. acidophilus* group.

In view of the foregoing, the aim of this work was to study the growth of strain K1 in various media, to assay the enzymes of carbon metabolism as a function of cultivation conditions, and to evaluate the effect of CO_2 on the growth parameters and enzymatic activities of this strain.

MATERIALS AND METHODS

The thermotolerant strain K1 was isolated from oxidized lead–zinc ores of Uzbekistan [4]. The strain was grown in Manning medium [10] under auto-, mixo-, and heterotrophic conditions. Under autotrophic conditions, the medium was supplemented with 1 mM $Na_2S_2O_3$ and either 10 g/l pyrite or 70 mM $FeSO_4 \cdot 7H_2O$ as the source of energy. Under mixotrophic conditions, the medium was additionally supplemented with 0.2 g/l glucose and yeast extract. Under organotrophic conditions, the medium was supplemented either with 0.5 g/l yeast extract or with 0.2 g/l each of glucose and yeast extract. The strain was grown at 37–40°C at an aeration rate of 2 volumes of air per 1 volume of the medium per min. In some experiments, the medium was enriched in CO_2 by bubbling the air with an elevated content of CO_2 (5 vol %).

To assay enzymes, bacterial cells from the exponential growth phase were separated from the medium by centrifugation at 10000 *g*, washed several times with the medium containing no energy source and then with 0.05 M Tris–HCl buffer (pH 7.4), suspended in double-strength buffer, and broken by ultrasonic treatment at 22 kHz for a total of 6 min (six 1-min bursts with breaks for cooling). The homogenate was centrifuged at 40000 *g* for 30 min, and the supernatant was assayed for enzymatic activities at 40–45°C in 0.1 M Tris–HCl buffer (pH 7.4). The enzymes of carbohydrate metabolism and the TCA cycle were assayed spectrophotometrically, using a Hitachi-200-20 spectrophotometer (Japan) [8, 9]. The activities of ribulose bisphosphate (RuBP) carboxylase and other carboxylases were measured by the radiometric method [11].

The protein concentration in the cell biomass and cell-free extracts was determined by the method of Lowry *et al.* [12]. Ferrous ions and glucose were determined, respectively, with the complexone Trilon B and colorimetrically, as described earlier [3].

RESULTS

1. Growth of Strain K1

The thermotolerant strain K1 grew well under mixotrophic conditions and could grow over several passages under autotrophic and heterotrophic conditions.

Upon mixotrophic growth in the medium with thiosulfate and either ferrous ions or pyrite in the presence of 0.2 g/l each of yeast extract and glucose, strain K1 exhibited a doubling time of 3 h. In this case, the biomass yield was 20–23 mg protein/l, whereas it was only 7–8 and 15–18 mg protein/l, respectively, during the first passages of autotrophic and heterotrophic growth. During mixotrophic growth, strain K1 oxidized up to 14 mM Fe^{2+} (about 20% of the initial content) and up to 0.9 mM glucose (about 80% of the initial content). The mixotrophic culture was dominated by medium-sized cells ($3.0–4.0 \times 0.9–1.0$ μm) with a certain number of large cells ($6.0–7.0 \times 0.9–1.0$ μm).

Under heterotrophic conditions, strain K1 grew with a doubling time of 5–5.5 h. The culture was dominated by medium-sized cells and contained prespores and spores. The residual content of glucose in the medium reached 35–40% of its initial content.

The autotrophic growth of strain K1 in the medium with Fe^{2+} or pyrite was characterized by doubling times of 18 and 20 h, respectively. Cells were small ($1.0–2.0 \times 0.7–0.8$ μm). The amount of the ferrous ions oxidized was low (15–20% of the initial content). In the autotrophic medium saturated with CO_2, strain K1 retained its polymorphism with a tendency toward reduction in the cell size.

2. Enzymes of the TCA Cycle

Strain K1 was found to have all enzymes of the TCA cycle and one key enzyme of the glyoxylate cycle, malate synthase (Table 1). The other key enzyme of the glyoxylate cycle, isocitrate lyase, was not detected in this strain, whatever cultivation conditions were used. The enzymatic activities depended on cultivation conditions. The key enzyme of the TCA cycle (citrate synthase), as well as aconitate hydratase and isocitrate dehydrogenase, was most active in cells grown under mixotrophic conditions, especially when Fe^{2+} was used as the electron donor. The activities of citrate synthase, aconitate hydratase, isocitrate dehydrogenase, and malate dehydrogenase in cells grown in the presence of pyrite (data not shown) were lower than in cells grown in the presence of Fe^2 and thiosulfate. This can be

Table 1. Activities of enzymes of the TCA and glyoxylate cycles in strain K1 grown under different cultivation conditions at different levels of CO_2 in the air used for aeration of the medium

Enzyme	Cultivation conditions				
	Autotrophic		Mixotrophic		Heterotrophic
	atmospheric level of CO_2	5 vol % CO_2	atmospheric level of CO_2	5 vol % CO_2	atmospheric level of CO_2
Citrate synthase (EC 4.1.3.7)	24.2	20.4	41.7	18.4	5.1
Aconitate hydratase (EC 4.2.1.3)	352.4	180.7	405.2	214.3	153.2
Isocitrate dehydrogenase (EC 1.1.1.4)	114.6	48.5	215.7	194.6	122.0
2-Oxoglutarate dehydrogenase (EC 1.2.4.2)	29.2	23.3	29.5	35.7	8.5
Succinate dehydrogenase (EC 1.3.99.1)	91.9	141.2	84.4	82.0	90.6
Fumarate hydratase (EC 4.2.1.2)	320.3	378.5	132.3	277.1	160.3
Malate dehydrogenase (EC 1.1.1.37)	250.7	295.6	166.8	590.3	151.4
Malate synthase (EC 4.1.3.2)	24.2	22.1	15.9	20.4	10.9

Note: Enzymatic activities are given in nmol/(min mg protein).

Table 2. Activities of enzymes of carbohydrate metabolism in strain K1 grown under different cultivation conditions at different levels of CO_2 in the air used for aeration of the medium

Enzyme	Cultivation conditions		
	Mixotrophic		Heterotrophic with yeast extract
	atmospheric level of CO_2*	5 vol % CO_2	atmospheric level of CO_2
Hexokinase (EC 2.7.1.1)	43.5	93.4	7.2
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	143.3	180.2	87.6
6-Phosphogluconate dehydrogenase (EC 1.1.1.44)	10.0	16.6	ND
6-Phosphogluconate dehydratase (EC 4.2.1.12) + 2-Keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14)	76.3	20.8	23.9
Fructose bisphosphate aldolase (EC 4.1.2.13)	705.0	24.6	20.8
Phosphofructokinase (EC 2.7.1.11)	125.2	250.1	8.9
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	183.3	129.1	7.3
Pyruvate kinase (EC 2.7.1.40)	10.2	45.8	ND

Note: Enzymatic activities are given in nmol/(min mg protein).

* Data of Karavaiko *et al.* [3]. ND stands for "not detected."

explained by the fact that pyrite is a recalcitrant substrate.

The activity of citrate synthase in cells grown organotrophically was lower than in cells grown mixotrophically (Table 1). The activities of aconitate hydratase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, i.e., the enzymes that catalyze the initial reactions of the TCA cycle, in organotrophically grown cells were also lower than in mixotrophically grown cells, whereas the activities of enzymes catalyzing the subsequent reactions of the TCA cycle were about the same in these two kinds of cells.

The activities of citrate synthase, aconitate hydratase, and isocitrate dehydrogenase in autotrophically grown cells were lower than in cells grown mixotrophically, i.e., under the most favorable conditions (Table 1). At the same time, fumarate hydratase, malate dehydrogenase, and malate synthase were more active in autotrophic than in mixotrophic or heterotrophic cells.

The CO_2 enrichment of the mixotrophic medium supplemented with Fe^{2+} and thiosulfate diminished the activities of citrate synthase and aconitate hydratase and enhanced the activities of fumarate hydratase and malate dehydrogenase (Table 1). The increased concentration of CO_2 in the autotrophic medium led to a decrease in the activities of aconitate hydratase and isocitrate dehydrogenase in cells of strain K1.

Table 3. Activities of various carboxylases in strain K1 grown under different cultivation conditions at different levels of CO_2 in the air used for aeration of the medium

Enzyme	Cultivation conditions				
	Autotrophic		Mixotrophic with glucose		Heterotrophic with glucose
	atmospheric level of CO_2	5 vol % CO_2	atmospheric level of CO_2	5 vol % CO_2	atmospheric level of CO_2
RuBP carboxylase (EC 4.1.1.39)	4.3	1.3	2.1	0.6	ND
Pyruvate carboxylase (EC 6.4.1.1)	1.4	0.8	0.46	0.15	ND
PEP carboxylase (EC 4.1.1.31)	3.5	0.4	1.52	0.64	1.34
PEP carboxykinase (EC 4.1.1.32)	0.6	0.1	ND	ND	ND
PEP carboxytransphosphorylase (EC 4.1.1.38)	3.1	0.9	0.92	0.54	ND

Note: Enzymatic activities are given in nmol/(min mg protein). ND stands for "not detected."

3. Enzymes of Carbohydrate Metabolism

Strain K1 contains enzymes of all three major pathways of carbohydrate metabolism [3]. Cells grown organotrophically, i.e., in the presence of glucose and/or yeast extract as the sources of carbon and energy, contain the key enzymes of two catabolic pathways, the Embden–Meyerhof–Parnas and the Entner–Doudoroff pathways (see Table 2 and [3]). In cells grown in the presence of yeast extract alone, the activities of these enzymes were 3 to 6 times lower than in cells grown organotrophically in the presence of glucose (Table 2). Cells of strain K1 grown mixotrophically contained not only the enzymes of the two aforementioned pathways, but also 6-phosphogluconate dehydrogenase [3]. This fact may indicate the involvement of the pentose phosphate pathway in the glucose metabolism of strain K1. Earlier, Smith *et al.* [13] showed the operation of all three major pathways in another lithotrophic microorganism, *Thiobacillus* A2 (presently *Paracoccus versutus* [14]).

In cells grown mixotrophically in the presence of elevated concentrations of CO_2, the activities of hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase increased (Table 2) and the activities of 6-phosphogluconate dehydratase, 2-keto-3-deoxy-6-phosphogluconate aldolase, and fructose bisphosphate aldolase decreased as compared with cells grown at the atmospheric content of CO_2. These data suggest that increased amounts of CO_2 in the medium activate the pentose phosphate pathway of glucose metabolism. The saturation of autotrophic medium with CO_2 somewhat enhanced the activities of fructose bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase, which can be associated with a more intense synthesis of carbohydrates in strain K1 grown under these conditions.

4. Carboxylases

The activity of the key enzyme of the Calvin cycle (RuBP carboxylase) in cells grown autotrophically in the presence of Fe^{2+} was about 2 times higher than in cells grown mixotrophically in the presence of glucose and Fe^{2+} (Table 3).

In the organotrophically grown strain K1, the activity of RuBP carboxylase was not detected, whereas the moderately thermophilic sulfobacilli *S. acidophilus* strain ALV and *S. thermosulfidooxidans* strains 1269^T and 41 possess active RuBP carboxylase whatever cultivation conditions are used [9, 15, 16]. The autotrophically and mixotrophically grown cells of strain K1 were found to contain pyruvate carboxylase, whose activity was 3 to 4 times higher in cells grown autotrophically at the atmospheric content of CO_2 than in cells grown mixotrophically (Table 3). In cells grown organotrophically, pyruvate carboxylase was not detected.

Phosphoenolpyruvate (PEP) carboxylase was the only enzyme whose activity was found in strain K1 irrespective of growth conditions (Table 3). The activity of PEP carboxykinase was detected only in autotrophically grown cells. PEP carboxytransphosphorylase was detected both in cells grown autotrophically and in those grown mixotrophically, the activity of this enzyme being maximum under autotrophic conditions at the atmospheric content of CO_2 (Table 3).

Elevated concentrations of CO_2 led to a decrease in the activity of all the investigated carboxylases under both autotrophic and mixotrophic growth conditions (Table 3).

DISCUSSION

The constructive metabolism of a large group of chemolithotrophic bacteria belonging to the genus *Sulfobacillus* is insufficiently studied, except for the investigations of Wood and Kelly performed with *S. acidophilus* strain ALV [15] and the later investigations of Karavaiko *et al.* performed with the type strain 1269

and the asporogenic mutant strain 41 of *S. thermosulfidooxidans* [3, 8, 9, 16]. It has been found that sulfobacilli grow best in mineral media with inorganic sources of energy in the presence of some amounts of organic substrates [4, 6, 7, 18]. Like strains 1269^T and 41, strain K1 can grow during several passages under autotrophic and heterotrophic conditions, albeit at a slower rate and with a lower biomass yield than under mixotrophic conditions. The autotrophic and heterotrophic growth of strain K1 results in changes in the morphology of cells, enhanced tendency to pleomorphism, and reduced activity of some enzymes of carbon metabolism as compared with growth under mixotrophic conditions.

Like strains 1269^T and 41 grown under auto-, mixo-, and heterotrophic conditions [9, 16], strain K1 grown autotrophically and mixotrophically contains RuBP carboxylase and some other carboxylases, whose activity depends on the growth conditions of the strain. Mixotrophic conditions partially and heterotrophic conditions completely inhibit the activity of RuBP carboxylase in strain K1. These data are in agreement with those of Smith *et al.* [13], who showed that the activity of pyruvate carboxylase in a chemostat culture of *Thiobacillus* A2 is 4 to 5 times lower under heterotrophic than under autotrophic or mixotrophic conditions. In contrast, high activity of PEP carboxylase was detected only in heterotrophically grown cells.

Under autotrophic conditions, strain K1 grows more poorly than the other sulfobacilli studied [9, 15, 16], likely because of the low activity of enzymes involved in the autotrophic fixation of carbon dioxide and the limited ability of this strain to oxidize inorganic sources of energy. However, strain K1 grown heterotrophically in the presence of glucose or yeast extract shows better growth and a higher biomass yield, as well as has more active enzymes of glycolysis and the TCA cycle, than do the moderately thermophilic sulfobacilli. In these parameters, strain K1 resembles *S. disulfidooxidans* SD-11, which also grows well on carbohydrates [7].

In strain K1, the TCA cycle is fully active under all growth conditions, whereas in other *S. thermosulfidooxidans* strains studied (1269^T and 41), the TCA cycle is blocked at the level of 2-oxoglutarate dehydrogenase [8, 9].

The presence of the complete TCA cycle, carboxylases, and the enzymes of carbohydrate metabolism allows strain K1 to efficiently use organic compounds. In other words, strain K1 is more chemoorganoheterotrophic than the other sulfobacilli studied [3].

Nevertheless, the organotrophic growth of strain K1, as well as of other sulfobacilli, is likely restricted by a deficiency of energy necessary for the maintenance of metabolism and the transport of organic compounds into cells. Furthermore, the absence or low activity of carboxylating enzymes, pyruvate kinase, and the glyoxylate cycle enzymes in strain K1 grown under heterotrophic conditions do not permit this strain to replenish the metabolites that are necessary for biosynthetic purposes.

It is known that thermophiles often suffer from a shortage of CO_2, especially at low pH values. An elevated content of CO_2 in the air used for aeration provides for a more active oxidation of iron under autotrophic conditions [15] and enhances the activity of enzymes responsible for the initial reactions of the TCA cycle in *S. thermosulfidooxidans* strains 1269^T and 41 [9]. In contrast, the activity of these enzymes in strain K1 grown autotrophically at an elevated content of CO_2 in the air was found to be low. The activity of RuBP carboxylase under these conditions was also low. A similar effect observed in *Thiobacillus* A2 and *Thiobacillus neapolitanus* [13, 19] was tentatively ascribed by the authors to a repressive action of PEP.

The activity of other carboxylating enzymes in strain K1 grown either autotrophically or mixotrophically at an elevated content of CO_2 in the air was also low (Table 3). This strain seems not to suffer from CO_2 limitation even when air with an atmospheric content of CO_2 is used for intense aeration of the growth medium.

The activities of the carbohydrate metabolism enzymes in strain K1 differ from those in the other *S. thermosulfidooxidans* strains studied (1269^T and 41) [3, 8, 9] and in *S. acidophilus* ALV, in which glucose is metabolized by the pentose phosphate pathway [15]. In the growth rate and biomass yield, the thermotolerant strain K1 can be ascribed neither to the *S. thermosulfidooxidans* group nor to the *S. acidophilus* group [3, 6].

The sulfobacilli under consideration also differ in some other biochemical, morphological, and physiological properties [2–4, 8, 20], as well as in the 16S rRNA sequences and the degree of DNA–DNA hybridization [2]. For instance, the degree of DNA homology of strain K1 with *S. acidophilus* NAL and *S. disulfidooxidans* SD-11 is only 5 and 22%, respectively. In general, the physiological and biochemical differences of strain K1 from the moderately thermophilic strains 1269^T and 41 correlate with their genotypic differences at the species level.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, grant nos. 01-04-48371 and 00-15-97765, and by the Russian Federal Scientific and Technical Program "The Biotechnology of the Extraction and Processing of Minerals," grant no. 43.073.1.1.2515.

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