Microbiology, Vol. 70, No. 3, 2001, pp. 245–250. Translated from Mikrobiologiya, Vol. 70, No. 3, 2001, pp. 293–299.

EXPERIMENTAL ARTICLES

Growth and Carbohydrate Metabolism of Sulfobacilli

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Received June 21, 2000

Abstract—The moderately thermophilic acidophilic bacteria *Sulfobacillus thermosulfidooxidans*, strain 1269, *S. thermosulfidooxidans* subsp. "*asporogenes*," strain 41, and the thermotolerant strain *S. thermosulfidooxidans* subsp. "*thermotolerans*" K1 prefer mixotrophic growth conditions (the concomitant presence of ferrous iron, thiosulfate, and organic compounds in the medium). In heterotrophic and autotrophic growth conditions, these sulfobacilli can grow over only a few culture transfers. In cell-free extracts of these sulfobacilli, key enzymes of the Embden–Meyerhof–Parnas, pentose-phosphate, and Entner–Doudoroff pathways were found. The role of a particular pathway depended on the cultivation conditions. All of the enzymes assayed were most active under mixotrophic conditions in the presence of Fe^{2+} and glucose, suggesting the operation of all of the three major pathways of carbohydrate metabolism under these conditions. However, the operation of the Entner–Doudoroff pathway in strain 41 was restricted under mixotrophic conditions. After the first culture transfer from mixotrophic to heterotrophic conditions, the utilization of glucose occurred only via the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways. After the first culture transfer from mixotrophic to autotrophic conditions, the activity of carbohydrate metabolism enzymes decreased in all of the strains studied; in strain K1, only the glycolytic pathway remained operative. The high activity of fructose-bisphosphate aldolase, remaining in strain 41 cells under these conditions, suggests the involvement of this enzyme in the reactions of the Calvin cycle or of gluconeogenesis.

Key words: sulfobacilli, enzymes of carbohydrate metabolism, pathways of carbohydrate metabolism, growth conditions.

The aerobic acidophilic bacteria of the genus *Sulfobacillus* belong to mixotrophic organisms that grow on media containing both inorganic (reduced iron and sulfur compounds, sulfide minerals) and organic compounds (yeast extract, casein hydrolysate, peptone, some sugars and amino acids) [1–6]. In heterotrophic conditions, sulfobacilli grow in the presence of ferrous iron (10 mg/l) on medium with 0.02% yeast extract; in autotrophic conditions, in the presence of reduced sulfur compounds (0.15 g/l of thiosulfate or tetrathionate) or yeast extract (0.002%), which are added to the medium along with ferrous iron.

Norris *et al.* [6] arbitrarily subdivided *Sulfobacillus*-like moderately thermophilic bacteria into two groups; this subdivision was based on a number of criteria, including the parameters of autotrophic growth with iron and sulfur and the growth rate and cell biomass yield under heterotrophic conditions. Strains of the species *S. thermosulfidooxidans* were assigned to the first group, and strains of the species *S. acidophilus* were assigned to the second group. Under autotrophic conditions, strains belonging to the first group oxidize ferrous iron at a higher rate than strains of the second group and are resistant to the inhibitory effect of ferric oxide. Only strains of the second group can grow on medium with sulfur over an unlimited number of culture transfers; the growth of sulfobacilli of the first group in the absence of yeast extract becomes weaker over culture transfers, and these cultures cannot be maintained for a long time [6, 7]. Strains of the second group also show a higher growth rate under heterotrophic conditions; their biomass yield on medium with 0.025% yeast extract and 10 mg/l of ferrous iron is about 30% higher than that of strains of the first group.

We have already demonstrated earlier that, both under autotrophic and heterotrophic conditions, strain *S. thermosulfidooxidans* 1269^T grows over a few culture transfers [8]. It was also shown that in both *S. thermosulfidooxidans* 1269^T [8] and *S. thermosulfidooxidans* subsp. "*asporogenes*" strain 41 [9], the activities of several TCA cycle enzymes under heterotrophic conditions are considerably lower than under mixotrophic conditions or cannot be detected at all. Moreover, Wood and Kelly, who studied the physiology of strain ALV, which belongs to the second group, suggested that, under heterotrophic conditions, the pathways of glucose catabolism are characterized by low efficiency due to energy expenditures for glucose transport, which is coupled to iron oxidation [2]. Zakharchuk *et al.* [8] demonstrated the low activity of carbo-

0026-2617/01/7003-0245$25.00

hydrate metabolism enzymes in heterotrophically and autotrophically grown cultures of *S. thermosulfidooxidans* 1269. In heterotrophically grown cells, the oxidative pentose-phosphate pathway was lacking. Cells of strain ALV [2], subsequently assigned to the species *S. acidophilus* [6], exhibited activities of the pentose-phosphate pathway key enzymes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase); the key enzymes of other catabolic pathways could not be detected even in mixotrophically grown cells [2]. Other representatives of *Sulfobacillus* have not been studied with respect to the peculiarities of carbohydrate catabolism.

In this work, we studied the activity of carbohydrate metabolism enzymes in the sulfobacilli *S. thermosulfidooxidans* 1269, *S. thermosulfidooxidans* subsp. "*asporogenes*" 41, and *S. thermosulfidooxidans* subsp. "*thermotolerans*" K1 grown in mixotrophic, heterotrophic, and autotrophic conditions.

MATERIALS AND METHODS

The subjects of this study were the moderate thermophiles *S. thermosulfidooxidans* 1269^T and *S. thermosulfidooxidans* subsp. "*asporogenes*" 41 [10] and the thermotolerant strain *S. thermosulfidooxidans* subsp. "*thermotolerans*" K1 [3, 11]. Strains 1269 and K1 were grown on Manning medium [12], and strain 41 was grown on Brierley medium [13]. For heterotrophic cultivation, the basal mineral media were supplemented with 1.2 mM glucose and 0.02% yeast extract (medium 1); for mixotrophic cultivation, 70 mM ferrous iron, 1–2 mM thiosulfate, 1.2 mM glucose, and 0.02% yeast extract were added (medium 2); autotrophic cultivation was performed in the presence of 70 mM ferrous iron and 1–2 mM thiosulfate (medium 3) at an atmospheric content of carbon dioxide. The initial pH value of the media used to cultivate strains 1269 and 41 was 1.8–2.0, and that of the medium used to cultivate strain K1 was 2.4–2.5. Cultivation was performed at 48–50°C (strains 1269 and 41) or 37–38°C (strain K1) in 5-l flasks with aeration. The inoculum was introduced in an amount of 10 vol % (mixotrophic and heterotrophic cultivation) or 15 vol % (autotrophic cultivation). The inoculum for heterotrophic cultures was grown under mixotrophic conditions on medium 2 and then on medium 1 supplemented with 2 mM ferrous iron. The material used to inoculate medium 3 was grown first mixotrophically and then on medium 3 supplemented with 0.002% yeast extract. It had earlier been established that the addition of 0.002% yeast extract does not influence the level of autotrophic fixation of $^{14}CO_2$ [8, 14].

All experiments were run in at least 3–5 replicates.

For enzyme assays, bacterial cells grown to the mid- or late exponential phase were harvested by centrifugation (10000 *g*), washed several times with basal mineral medium and then with 0.05 M Tris–HCl buffer (pH 7.4), and ultrasonically disrupted (22 kHz, six 1-min sessions with 2-min intervals, under cooling). The supernatant obtained by centrifugation (40000 *g*, 30 min) of the homogenate was used for spectrophotometric (Hitachi-220 spectrophotometer) assays of carbohydrate metabolism enzymes [8, 15], all of which were performed in 0.1 M Tris–HCl buffer, pH 7.4, at a temperature of 45–50°C. The following enzymes were assayed: hexokinase (ATP–glucose phosphotransferase, EC 2.7.1.1), glucose-6-phosphate dehydrogenase (NADP-dependent, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (NADP-dependent, EC 1.1.1.44), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); 6-phosphogluconate dehydratase (EC 4.2.1.12) plus 2-keto-3-deoxy-6-phosphogluconate (3-deoxy-2-oxo-6-phosphogluconate) aldolase (EC 4.1.2.14), 6-phosphofructokinase (EC 2.7.1.11), and pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40). The enzyme activities were expressed in nmol/(min mg protein). Reaction mixtures contained 0.5–1.5 mg protein/ml.

Protein was determined by the method of Lowry *et al.* Iron was determined complexonometrically; glucose was determined colorimetrically as described earlier [4, 8].

RESULTS AND DISCUSSION

Growth

All of the strains studied exhibited the highest growth rate and cell yield under mixotrophic conditions with glucose present in the medium (Figs. 1a–1c). The specific growth rate reached values of 0.25, 0.28, and 0.29 h^{-1} in strains 41, K1, and 1269, respectively. The biomass increase occurred at the expense of the consumption of glucose as the carbon source (Figs. 2a–2c). Concurrently, Fe^{2+} oxidation occurred (at rates of 0.62, 2.6, and 2.8 mM/h for strains K1, 1269, and 41, respectively). The amount of glucose consumed by strains 41, 1269, and K1 was 25, 35, and 80% of its initial content. Under mixotrophic conditions, the growth of strain 41 relied on CO_2 fixation to a greater extent than the growth of strain 1269. Glucose is weakly assimilated by strain 41, and at a concentration of 0.3 mM and higher, it inhibits autotrophic assimilation of CO_2 in this strain [14].

After a change in the cultivation conditions from mixotrophic to heterotrophic in the presence of glucose and yeast extract in the medium (first culture transfer), strains 41, 1269, and K1 used 10, 20, and 60%, respectively, of the glucose initially present in the medium. All strains, especially the moderately thermophilic ones, showed weak growth under heterotrophic conditions. After a culture transfer from medium 1 containing 2 mM ferrous iron to medium 1 devoid of iron, the growth rate decreased to 0.06 and 0.09 h^{-1} in the moderately thermophilic strains 41 and 1269 and to 0.13 h^{-1} in the thermotolerant strain K1. The growth ceased after three to six culture transfers.

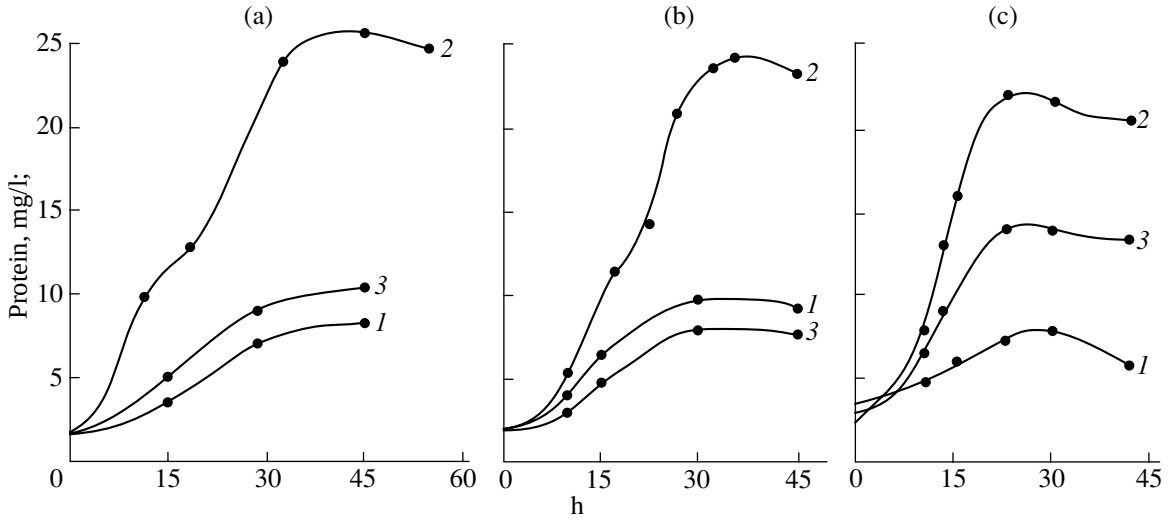

Fig. 1. Growth of *Sulfobacillus* strains (a) 1269, (b) 41, and (c) K1 under (*1*) autotrophic, (*2*) mixotrophic, and (*3*) heterotrophic conditions.

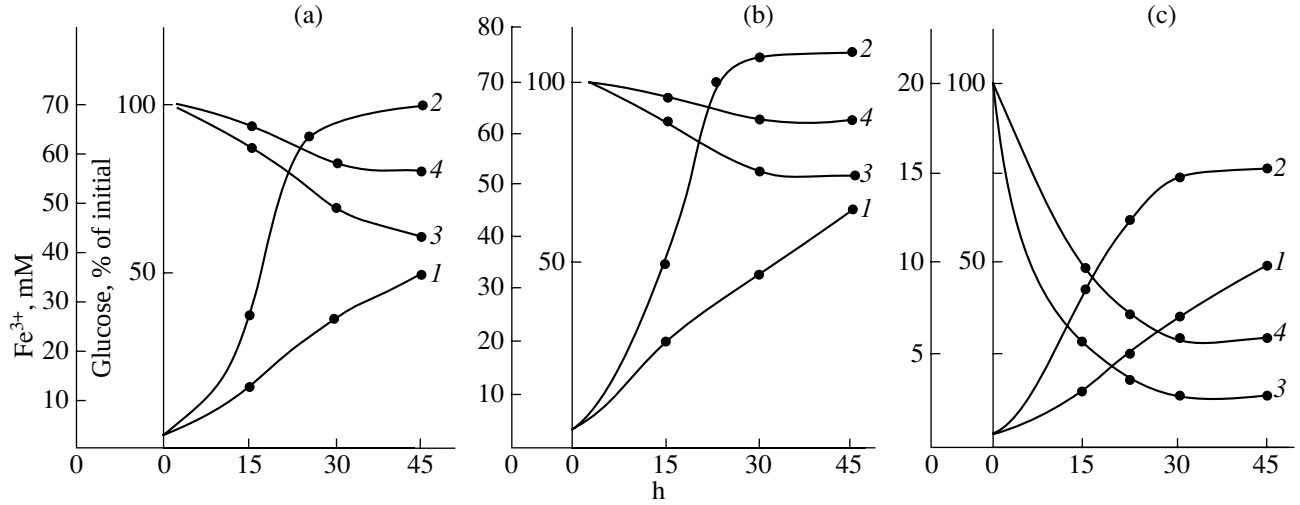

Fig. 2. Consumption of ferrous iron and glucose by *Sulfobacillus* strains (a) 1269, (b) 41, and (c) K1 under autotrophic and heterotrophic conditions: (*1*) Fe^{3+} formation under autotrophic conditions; (*2*) Fe^{3+} formation under mixotrophic conditions; (*3*) glucose consumption under mixotrophic conditions; (*4*) glucose consumption under heterotrophic conditions.

It has been shown that another representative of sulfobacilli, *S. acidophilus* ALV, can grow with glucose as the sole carbon and energy source; however, the growth was weak, and only one-fourth of the initial glucose amount was consumed [2].

As compared to moderate thermophiles, strain K1 shows a higher capacity for glucose utilization under heterotrophic conditions (see above). In this respect, it is similar to *S. disulfidooxidans* SD-11, which is capable of rapid growth on medium with glucose [5].

After a change in the cultivation conditions from mixotrophic to autotrophic (Fig. 1), strains 41, 1269, and K1 exhibited low growth rates already in the first culture transfer (0.035, 0.07, and 0.09 h^{-1}, respectively), as was the case with the culture transfer to heterotrophic conditions. The rate of iron oxidation was the highest in strain 41 (1 mM/h); in strains 1269 and K1, the rates of iron oxidation were 0.8 and 0.27 mM/h, respectively. The thermotolerant strain oxidized only 20–30% of the ferrous iron added to the medium.

To achieve a deeper understanding of the complicated physiology of the growth of sulfobacilli, we undertook an investigation of the enzymes of glucose metabolism.

Carbohydrate Metabolism Enzymes

When cultivated under different conditions, sulfobacilli exhibit different levels of the key enzymes of the three major pathways of carbohydrate catabolism.

Under the action of hexokinase (Tables 1, 2), glucose-6-phosphate is produced, which can be further metabolized via the Embden–Meyerhof–Parnas pathway (the key enzymes are phosphofructokinase and fructose-bisphosphate aldolase), pentose-phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), and Entner–Doudoroff pathway (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase).

During the growth of sulfobacilli under mixotrophic conditions in the presence of glucose and ferrous iron, the activities of a number of carbohydrate metabolism enzymes considerably differed from their activities recorded in cells grown under heterotrophic conditions in medium 1 with glucose (Tables 1, 2). Thus, in cells of strain 41 grown under mixotrophic conditions, the activities of hexokinase and glucose-6-phosphate dehydrogenase were 5 times higher, and the activity of fructose-bisphosphate aldolase was 10 times higher; the activity of 6-phosphogluconate dehydrogenase could also be detected. On the other hand, the activities of the key enzymes of the Entner–Doudoroff pathway decreased abruptly. It can be inferred that, under mixotrophic conditions, the glucose utilization by strain 41 switches to the Embden–Meyerhof–Parnas and pentose-phosphate pathways.

Under mixotrophic conditions, the thermophilic strain 41 (Table 1) and strain 1269 which was studied earlier [8], did not exhibit significant differences in their enzyme activity levels (in contrast to the situation observed under heterotrophic conditions). Exceptions were 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, which exhibited much higher activities in strain 1269. Thus, strain 1269, which consumes larger amounts of glucose, exhibits under mixotrophic conditions the high level of enzyme activities necessary for the operation of the Entner–Doudoroff pathway. The high activity of fructose-bisphosphate aldolase, the enzyme of the Embden–Meyerhof–Parnas pathway, taken together with the low activity of phosphofructokinase, may indicate the involvement of aldolase (both in strain 1269 and 41) not only in sugar metabolism, but also in other metabolic pathways, for example, in the reductive pentose-phosphate cycle. Indeed, the activity of ribulose-bisphosphate carboxylase (RuBPC) has been detected in cells of the moderately thermophilic strains 1269 and 41 grown mixotrophically [7, 9]. In addition, most of the TCA cycle enzymes, especially those involved in the initial reactions of the cycle, exhibit higher activities under mixotrophic conditions [8, 9]. These data can explain the fact that the growth of moderately thermophilic sulfobacilli is most active under mixotrophic conditions. Moderately thermophilic sulfobacilli can simultaneously gain energy from inorganic and organic substrates and assimilate organic carbon and carbon dioxide. The enzymes of carbohydrate metabolism, sufficiently active under mixotrophic conditions, play a role in these processes.

In cells of the thermotolerant strain K1 grown mixotrophically in the presence of glucose, the activities of hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase were as high as in heterotrophically grown cells (Table 2). However, the activities of the key enzymes of the Embden–Meyerhof–Parnas pathway (fructose-bisphosphate aldolase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase) were several times higher than in heterotrophically grown cells. Therefore, it can be assumed that glucose dissimilation in strain K1 cells grown mixotrophically occurs not only via the Entner–Doudoroff and pentose-phosphate pathways, but mainly via the glycolytic pathway.

Upon a change in the cultivation conditions from mixotrophic to heterotrophic, the activity of most enzymes decreases in the cells of sulfobacilli already after the first culture transfer (Tables 1, 2). Certain changes in the pathways of glucose metabolism also occur.

Judging from the enzyme activities, during heterotrophic growth on medium with glucose, the utilization of glucose by all the strains studied in this work occurs via the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways (Tables 1, 2) [8]. 6-Phosphogluconate dehydrogenase cannot be detected, suggesting that the pentose-phosphate pathway is not involved in the glucose metabolism under heterotrophic conditions.

Under heterotrophic conditions, strain K1 cells exhibit higher activities of a number of carbohydrate metabolism enzymes (Table 2) than strains 41 (Table 1) and 1269 [8]. The Entner–Doudoroff pathway in the thermotolerant strain is evidently more active than glycolysis, although the activity level of the enzymes of the latter pathway is also rather high.

Autotrophically grown cells of strain 41 (first culture transfer) exhibited the activities of all key enzymes of the Embden–Meyerhof–Parnas, Entner–Doudoroff, and pentose-phosphate pathways; however, these activities were lower than in cells grown mixotrophically in the presence of glucose and iron (Table 1). It should be noted that the activity of fructose-bisphosphate aldolase in autotrophically grown cells was considerably higher than in the cells grown heterotrophically with glucose. This fact, taken together with the low activity of phosphofructokinase (the other key enzyme of glycolysis), suggests the possible involvement of aldolase in the reactions of the Calvin cycle or gluconeogenesis.

As we showed earlier, the activities of carbohydrate metabolism enzymes in the cells of strain 1269 grown autotrophically are by about 40% lower than in cells grown mixotrophically in the presence of glucose (the exception is glyceraldehyde-3-phosphate dehydrogenase) [8]. In cells of strain 1269 grown autotrophically, we did not detect the activity of phosphofructokinase or

Table 1. Activities of carbohydrate metabolism enzymes (nmol/(min mg protein)) in cells of *S. thermosulfidooxidans* subsp. "asporogenes" strain 41 grown under various conditions

Enzyme	Cultivation conditions		
	autotrophic	heterotrophic (with glucose)	mixotrophic (with glucose)
Hexokinase	13.8	5.6	28.6
Glucose-6-phosphate dehydrogenase	13.8	26.8	101.2
6-Phosphogluconate dehydrogenase	3.1	0	4.2
6-Phosphogluconate dehydratase + 2-keto-3-deoxy-6-phosphogluconate aldolase	1.5	22.8	0.9
Fructose-bisphosphate aldolase	424.0	63.2	640.0
Phosphofructokinase	1.0	5.9	4.5
Glyceraldehyde-3-phosphate dehydrogenase	15.4	26.6	94.6
Pyruvate kinase	0	5.6	10.9

Table 2. Activities of carbohydrate metabolism enzymes (nmol/(min mg protein)) in cells of *S. thermosulfidooxidans* subsp. "*thermotolerans*" strain K1 and *S. thermosulfidooxidans* strain 1269 grown under various conditions

Enzyme	Cultivation conditions			
	strain K1			strain 1269
	autotrophic	heterotrophic (with glucose)	mixotrophic (with glucose)	mixotrophic (with glucose)
Hexokinase	5.0	42.2	43.5	68.0
Glucose-6-phosphate dehydrogenase	38.2	117.0	143.3	188.0
6-Phosphogluconate dehydrogenase	0	0	10.0	5.2
6-Phosphogluconate dehydratase + 2-keto-3-deoxy-6-phosphogluconate aldolase	0	64.4	76.1	128.0
Fructose-bisphosphate aldolase	30.9	28.7	705.0	464.0
Phosphofructokinase	1.7	63.7	125.2	8.7
Glyceraldehyde-3-phosphate dehydrogenase	35.8	10.8	183.3	135.0
Pyruvate kinase	8.8	0	10.2	11.2

enzymes of the pentose-phosphate pathway. Despite the presence of RuBPC and phosphoenolpyruvate carboxytransphosphorylase [7], the lack of the above-mentioned carbohydrate metabolism enzymes, together with the open TCA and low enzyme activities [8, 9], does not allow sulfobacilli to show stable growth under autotrophic conditions.

In cells of the thermotolerant strain K1, the activity of all the assayed enzymes sharply decreased after a change in cultivation conditions from mixotrophic to autotrophic. Moreover, only enzymes of the Embden–Meyerhof–Parnas pathway could be detected, whereas the key enzymes of the Entner–Doudoroff and pentose-phosphate pathways (6-phosphogluconate dehydratase, 2-keto-3-deoxy-6-phosphogluconate aldolase, 6-phosphogluconate dehydrogenase) could not.

Thus, the data obtained demonstrate that the strains studied utilize glucose via three major metabolic routes: the Embden–Meyerhof–Parnas, pentose-phosphate, and Entner–Doudoroff pathways. The relative importance of each pathway in vivo is unknown.

Preferential operation of one or another pathway of carbohydrate metabolism depends on the growth conditions. In heterotrophic conditions, the utilization of glucose by sulfobacilli may occur via the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways, whereas in mixotrophic conditions, the pentose-phosphate pathway may also be involved. During autotrophic growth, only the asporogenous strain 41 exhibits activities of the enzymes of all three major pathways (however, their activities are low); cells of strain 1269 lack key enzymes of the pentose-phosphate pathway, and cells of strain K1 lack key enzymes of both pentose-phosphate and Entner–Doudoroff pathways.

The activity of all enzymes sharply decreases under autotrophic and heterotrophic conditions; thus, such

conditions should be considered inappropriate for the growth of at least some sulfobacilli.

The highest protein yield observed in the cells of sulfobacilli under mixotrophic conditions, together with the highest activity of most carbohydrate metabolism enzymes under these conditions, allow an assumption to be made that sulfobacilli can use the additional energy derived from glucose oxidation during lithoheterotrophic growth. Under the latter conditions, the growth is stable. A higher capacity for glucose utilization is characteristic of the thermotolerant strain K1 and the mesophilic strain SD-11 [5]; it is also manifest in these strains under heterotrophic conditions. The taxonomic status of strain K1 should evidently be reconsidered [15].

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project nos. 98-04-48158 and 00-15-97765.

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