Microbiology, Vol. 72, No. 5, 2003, pp. 558–563. Translated from Mikrobiologiya, Vol. 72, No. 5, 2003, pp. 627–632.

EXPERIMENTAL ARTICLES

Carbohydrate Metabolism of the Saccharolytic Alkaliphilic Anaerobes *Halonatronum saccharophilum*, *Amphibacillus fermentum*, and *Amphibacillus tropicus*

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Received July 22, 2002

Abstract—The saccharolytic anaerobic bacteria *Halonatronum saccharophilum*, *Amphibacillus fermentum*, and *Amphibacillus tropicus* produce formate, the main fermentation product. In the alkaliphilic community, formate is used as the preferential substrate for sulfate reduction. To reveal the pathways of carbohydrate fermentation by these bacteria, the activity of the key enzymes of carbohydrate metabolism and their pH dependence was studied. It was established that *H. saccharophilum* utilized glucose by the fructose bisphosphate and hexose monophosphate pathways, and *A. tropicus*, by the fructose bisphosphate and Entner–Doudoroff pathways. The activity of the key enzymes of all three pathways of glucose metabolism was detected in *Amphibacillus fermentum*. According to the data obtained, the glucose catabolism in *H. saccharophilum*, *A. fermentum*, and *A. tropicus* mainly proceeds via the fructose bisphosphate pathway. The pH optima of the key enzymes of the glucose metabolism of the alkaliphiles are shifted to alkaline values. In *A. tropicus*, formate is formed from pyruvate under the action of pyruvate formate-lyase; and in the haloanaerobe *H. saccharophilum*, formate dehydrogenase is involved in formate metabolism.

Key words: carbohydrate metabolism, enzyme activity, formate production, alkaliphiles, saccharolytics.

The extremely alkaliphilic microorganisms have been isolated from different habitats and are diverse with respect to their physiology and taxonomic position [1–3]. Among the saccharolytic bacteria inhabiting natural soda reservoirs, anaerobic bacilli have been the most studied as model objects for investigating the bioenergetics of alkaliphiles [4] and as sources of alkaline exoenzymes for industry [5]. Anaerobic alkaliphilic saccharolytic bacteria were not known [1], and only recently did they become subjects of study [3].

During the study of alkaliphilic bacteria involved in carbohydrate degradation, a number of saccharolytic microorganisms belonging to various taxonomic groups were isolated from the highly mineralized continental soda Lake Magadi (Kenya) under strictly anaerobic conditions [6, 7]. Among them, the first saccharolytic alkaliphile, the obligate anaerobe *Halonatronum saccharophilum* [8] belonging to the order *Halanaerobiales* (which mainly includes marine representatives), and *Amphibacillus fermentum* and *Amphibacillus tropicus*, facultatively anaerobic bacilli belonging to group I of the species spectrum of the genus *Bacillus*, were described [9]. These bacteria have a fermentive type of metabolism and utilize certain mono-, di-, and polysaccharides as the source of carbon and energy. Formate is one of their main products of glucose fermentation. In addition, all of them produce acetate, ethanol, and CO_2, and *H. saccharophilum* also forms H_2. In the trophic system of the anaerobic alkaliphilic community, these microorganisms are primary anaerobes interacting with both the bacteria of the first hydrolytic phase and with secondary anaerobes utilizing their metabolites. In this connection, it is important to understand the carbohydrate metabolism of the new isolates and the pathways of carbohydrate utilization operating in them.

To elucidate the pathways of carbohydrate utilization by the alkaliphilic saccharolytic bacteria *H. saccharophilum*, *A. fermentum*, and *A. tropicus*, the activity of the key enzymes of glucose catabolism and the pH-dependence of the activity of some of them were determined. Since formate is synthesized by these organisms in considerable amounts and is involved in the interspecies hydrogen transfer in the community, possible mechanisms of the formation of this product were studied in *H. saccharophilum* and *A. tropicus*.

MATERIALS AND METHODS

Bacterial strains and the conditions for their cultivation. The studies were conducted using the type strains of saccharolytic bacteria: the obligate anaerobe

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Halonatronum saccharophilum Z-7986^T = DSM13868 [8] and the facultative anaerobes *Amphibacillus fermentum* Z-7984^T = DSM13869 and *Amphibacillus tropicus* Z-7992^T = DSM13870 [9] isolated from the highly mineralized Lake Magadi (Kenya). All the cultures were grown at a temperature of 36°C, optimal for growth, in medium of the following composition (g/l): KH_2PO_4, 0.2; $MgCl_2$, 0.1; NH_4Cl, 0.5; KCl, 0.2; NaCl, 50.0; Na_2CO_3, 68.0; $NaHCO_3$, 38.0; $Na_2S \cdot 9H_2O$, 0.7; yeast extract, 0.2; glucose, 3.0; trace element solution, 1 ml/l; vitamin solution, 10 ml/l [8]; resazurin, 0.001; pH 9.75. Preparation of the medium and cultivation were carried out under strictly anaerobic conditions in an atmosphere of nitrogen. To avoid glucose caramelization under alkaline conditions, its sterile solution was introduced immediately before inoculation. A suspension of cells taken from the exponential growth phase served as the inoculum.

Analytical methods. Growth was assessed by measuring optical density in Hungate tubes at 600 nm on a Specol-10 spectrophotometer (Jena). Glucose was analyzed by the reaction with phenol [8]. Volatile fatty acids were determined using a 3700 gas chromatograph with a flame ionization detector. Formate was determined colorimetrically [8].

Methods for determining the enzyme activities. For the determination of enzyme activities, cells in the exponential growth phase were used. They were separated from the culture medium by centrifugation for 50 min at 4140 *g*. When the activity of the key enzymes of glucose metabolism was studied, the cells were washed with a 0.1 M Tris–HCl buffer, pH 8.5. When the activity of the enzymes involved in formate metabolism was determined, the cells were washed and resuspended in 50 mM of a KH_2PO_4 buffer (pH 7.8) containing 0.4 mM Mg^{2+} and 0.3 mM dithiothreitol. The cells were disrupted ultrasonically (22 kHz) in the corresponding buffer for 45 to 60 s under cooling or with X-PRESS (Type X-25, Biotec, Stockholm, Sweden). The supernatant obtained after centrifugation of the cell homogenate for 15 min at 40000 *g* was used further.

The activity of glucose metabolism enzymes was measured spectrophotometrically by monitoring NAD(H) or NADP(H) oxidation or reduction at 340 nm. Hexokinase (EC 2.7.1.1 ATP:D-hexose-6-phosphotransferase) was determined from NADP reduction in the presence of glucose, ATP, and glucose-6-phosphate dehydrogenase [10]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49 D-glucose-6-phosphate:NADP-oxidoreductase) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43 6-phosphogluconate:NADP-oxidoreductase) were determined from NADP reduction in the presence of glucose-6-phosphate and 6-phosphogluconate, respectively [10]. 6-Phosphogluconate dehydratase (EC 4.2.1.12 6-phospho-D-gluconate-hydro-lyase) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14 6-phospho-2-keto-3-deoxy-D-gluconate:D-glyceraldehyde-3-phosphate-lyase) were determined from NADH oxidation n the presence of 6-phosphogluconate and lactate dehydrogenase [11]. Phosphofructokinase (EC 2.7.1.11 D-fructose-6-phosphate-1-phosphotransferase) was determined from NADH oxidation in the presence of fructose-6-phosphate [12]. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12 glyceraldehyde-3-phosphate:NAD-oxidoreductase(phosphorylating)) was determined from NADH oxidation in the presence of 3-phosphoglyceric acid [12]. Fructose-1,6-4-bisphosphate aldolase (EC 4.1.2.13 fructose-1,6-diphosphate:D-glyceraldehyde-3-phosphate lyase) was determined colorimetrically from the formation of trioses from fructose bisphosphate at 540 nm [12].

Pyruvate formate lyase (EC 2.3.1.54) was determined from the formation of formate from pyruvate spectrophotometrically at 515 nm [13]. The reaction mixture was placed into Tunberg tubes from which the gas phase was repeatedly evacuated to be replaced with molecular nitrogen. The reaction was stopped by heating the reaction mixture in a boiling water bath for 1 min. Formate in the supernatant was determined by the reaction with acetamide and citric acid [8]. Formate dehydrogenase was determined by monitoring, in the pressure the formate, the reduction of NADH and NADPH at 340 nm or the reduction of dichlorophenolindophenol (DCPP), phenazine methosulfate (PMS), or methyl viologen at 600 nm [14]. The reaction was carried out in anaerobic cuvettes from which air was evacuated to be replaced with nitrogen.

All measurements were carried out at room temperature using a Hitachi 200-20 spectrophotometer. Protein in extracts was determined by the Lowry method. The enzyme activity was expressed in nmol substrate/(min mg protein).

When studying the relationship between the fructose bisphosphate aldolase activity and the pH of the reaction mixture, we used acetate buffer with a ionic strength of 0.1 or 0.1 M Tris Base buffer. The pH was adjusted to required values by titration of the buffers with 10% HCl or 10% NaOH.

RESULTS

All the bacteria studied required yeast extract as a source of amino acids. When studying the dependence of growth on the concentrations of glucose and yeast extract (Table 1), we noted that in *H. saccharophilum*, as the glucose content varied from 1.0 to 4.0 g/l, the growth rate did not change when the amount of yeast extract was increased from 0.1 to 0.8 g/l. However, when 8.0 g/l glucose was introduced, a linear dependence of the growth rate on the amount of the amino acid source was observed. An increase in the glucose concentration content stimulated bacterial growth at any concentration of yeast extract. Thus, in the haloanaerobe, the requirement for the growth factor was met by its minimal content, and the limiting factor was the substrate concentration. For *A. fermentum*, an increase

Table 1. Relationship between the growth of *H. saccharophilum*, *A. fermentum*, and *A. tropicus* and the glucose and yeast extract concentrations in the medium

Yeast extract, g/l	*H. saccharophilum*				*A. fermentum*				*A. tropicus*			
	Glucose, g/l											
	1.0	2.0	4.0	8.0	1.0	2.0	4.0	8.0	1.0	2.0	4.0	8.0
	Optical density (λ = 600 nm) after 18 h of growth											
0.1	0.05	0.09	0.30	0.26	0.13	0.15	0.17	0.21	0.07	0.07	0.07	0.07
0.2	0.05	0.09	0.29	0.35	0.17	0.22	0.23	0.25	0.13	0.15	0.13	0.13
0.4	0.05	0.09	0.29	0.42	0.22	0.30	0.41	0.48	0.19	0.21	0.25	0.23
0.8	0.05	0.09	0.30	0.53	0.25	0.47	0.62	0.64	0.23	0.41	0.40	0.40

in the growth rate was noted with an increase in the content of both glucose and the source of amino acids and nitrogen. For this bacillus, the concentrations of glucose and the growth factor in the medium were not optimal, and therefore an increase in their concentration significantly stimulated growth. In *A. tropicus*, an increase in the amount of yeast extract led to a growth rate increase at different glucose contents in the medium. A change in the glucose concentration from 1.0 to 8.0 g/l at a fixed concentration of yeast extract did not influence the growth of the bacillus (Table 1). Thus, in *A. tropicus*, it was the yeast extract, not the glucose, that limited growth. The bacteria studied are fast-growing fermenting copiotrophic zymogens: their growth was not inhibited by high concentrations of the substrate and the amino acid source. The maximal growth rate for the bacteria studied was recorded at 8 g/l of glucose and 0.8 g/l of yeast extract.

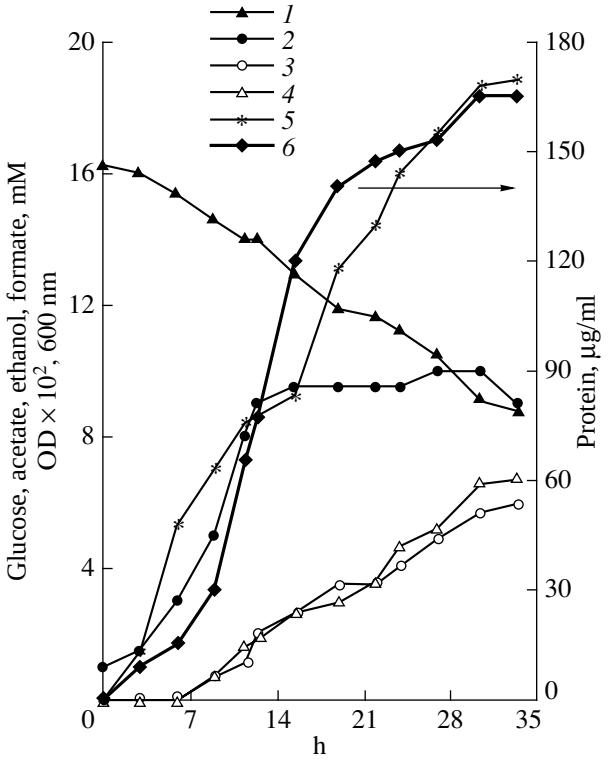

Fig. 1. Growth and production of volatile fatty acids by *H. saccharophilum* in medium with glucose: (*1*) glucose, mM; (*2*) OD × 10^2, λ = 600 nm; (*3*) acetate, mM; (*4*) ethanol, mM; (*5*) formate, mM; (*6*) protein, μg/ml.

Growth of *H. saccharophilum* in a medium with glucose. The standard medium for *H. saccharophilum* growth contained 17 mM glucose and 0.2 g/l of yeast extract. A short lag phase was noted in this medium, and the culture immediately passed to the exponential growth phase (Fig. 1). In the exponential growth phase, which lasted 17 h on average, a change in the optical density by one unit corresponded to a 1 mg/ml increase in the protein concentration. The generation time was 3.7 h. After 14 h of *H. saccharophilum* growth, the optical density curve reached its plateau, while the protein concentration continued to increase. The culture transition to spore formation, associated with active protein synthesis, seems to be the explanation for this phenomenon. After the stationary phase, whose average duration was 13 h, cell lysis occurred, and the culture cleared.

Throughout *H. saccharophilum* growth, the glucose concentration decreased gradually (Fig. 1). The substrate was used incompletely: of 16.3 mM glucose, 8.8 mM remained in the medium at the end of the stationary phase.

During the first 6 h after inoculation, no acetate or ethanol synthesis was noted, after which these products were formed in equal amounts up to the beginning of the stationary phase, and their final concentration in the medium constituted 5.9 and 6.7 mM, respectively (Fig. 1). Formate was the main product of glucose fermentation. Its presence in the medium was noted as quickly as 3 h after incubation, the final concentration attaining 19 mM.

Carbohydrate metabolism enzymes. The cells of all the three bacteria studied showed a high hexokinase activity (Table 2). In *Amphibacillus* representatives, it was equally high and several times higher than the activity exhibited by *H. saccharophilum*. In the cultures studied, glucose-6-phosphate dehydrogenase was also manifest; its activity in *A. tropicus* exceeded the activity in *A. fermentum* by a factor of 6. No activity of 6-phosphogluconate dehydrogenase, another key enzyme of

the hexose monophosphate (HMP) pathway, was revealed in *A. tropicus*, as distinct from *A. fermentum*. In *H. saccharophilum*, the activity of this enzyme was rather low. *A. tropicus* and *A. fermentum* contained the key enzymes of the Entner–Doudoroff pathway, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase. In *H. saccharophilum*, this pathway of sugar metabolism does not seem to function, since it did not exhibit the activity of the key enzymes. All the bacteria studied contained the main enzymes of the Embden–Meyerhof–Parnas pathway: phosphofructokinase, fructosebisphosphate aldolase, and glyceraldehydes-3-phosphate dehydrogenase, which had a sufficiently high activity.

To study the pH dependence of the activity of glucose metabolism enzymes, the cell extract of *A. tropicus* grown at pH 9.75 was used. This bacillus is an obligate alkaliphile growing at pH values between 8.5 and 11.5 and having an optimum pH at 9.5–9.7 [8]. The activity of glucose-6-phosphate dehydrogenase was observed in a wide pH range from 6.0 to 10.2 with a weakly pronounced optimum at pH 7.6–7.8 (Fig. 2a). The enzyme was inactive at pH 5.2 and 11.2, and the activity decreased abruptly to zero with a pH increase from 10.2 to 11.2. When determining the activity of fructose bisphosphate aldolase, we did not find pH values in the alkaline range at which the activity of this enzyme was zero (pH values up to pH 12.2 were studied). It increased smoothly from pH 4.5 to 8.3 to reach a plateau (Fig. 2b).

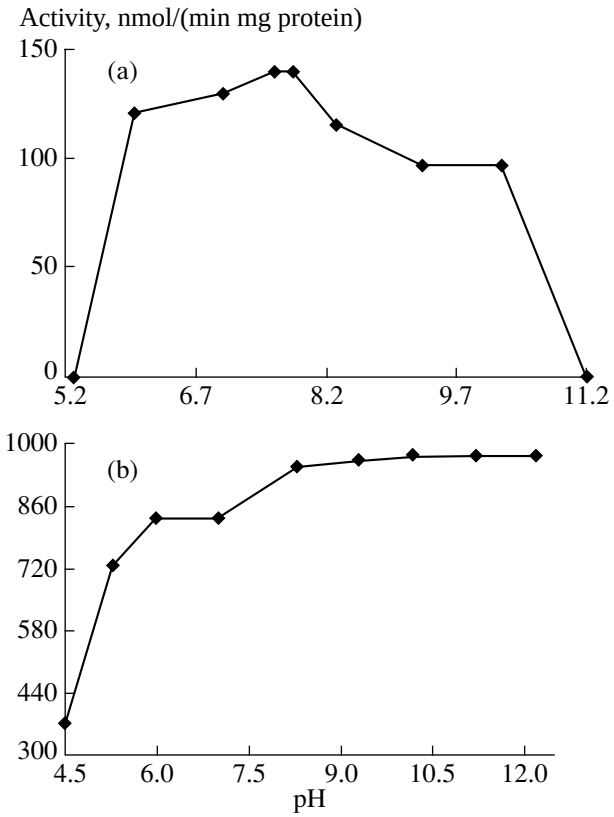

Fig. 2. pH dependence of the activities of (a) glucose-6-phosphatede hydrogenase and (b) fructosebisphosphate aldolase in the *A. tropicus* cell extract.

Enzymes involved in formate metabolism. One of the possible pathways of formate production in microorganisms is pyruvate cleavage under anaerobic conditions with the involvement of pyruvate formate lyase. In addition, under such anaerobic conditions formate can be synthesized via carbon dioxide reduction in a reaction catalyzed by formate dehydrogenase.

H. saccharophilum cells reveal formatedehydrogenase. The activity of this enzyme was rather low (Table 2) and could be detected only in the presence of the artificial electron acceptor methyl viologen. With DCPP and PMS, as well as with the natural electron acceptors NAD and NADH, we failed to reveal this activity. In *H. saccharophilum* cells, the activity of pyruvate formate lyase was not detected under the experimental conditions.

In contrast to *H. saccharophilum*, *A. tropicus* exhibited pyruvate formate lyase activity, although it was low. On the other hand, no formate dehydrogenase was revealed in this bacterium with any of the electron acceptors used.

DISCUSSION

All the bacteria studied are obligate or facultative anaerobes with a fermentative type of metabolism. As judged from the activity of the key enzymes of carbohydrate metabolism, *H. saccharophilum* can ferment glucose via the fructosebisphosphate (FBP) and HMP pathways. The Entner–Doudoroff pathway is nonfunctional in this haloanaerobe due to the absence of the activity of its key enzymes, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase. In glucose metabolism, *A. tropicus* uses the FBP pathway and the Entner–Doudoroff pathway. *A. fermentum* possesses the activity of the key enzymes of the three main pathways of glucose metabolism. Possibly, it is for this reason that it is able to ferment a wide spectrum of organic substrates, including pentoses, hexoses, sugar alcohols, di- and polysaccharides, esters, and nitrogenous compounds [9]. Despite the fact that the bacteria studied exhibited simultaneously the enzyme activity of several pathways of glucose catabolism, the FBP pathway enzymes were characterized by the highest activity (Table 2). It was shown for the representatives of the genus *Bacillus* that the FBP and HMP pathways are the most important for them [15]. In *B. thuringiensis* [16], *B. alvei*, *B. lentimorbus*, *B. popillae* [15], and *B. circulans* var. *alkaliphilus* [17], 75 to 94% of glucose is utilized during the exponential growth phase via the FBP pathway. The contribution of one or another pathway to overall carbohydrate metabolism depended on a number of factors, including the growth phase of the bacteria, aeration, the source of nitrogen, and temperature. The presence of yeast

Table 2. Activity of carbohydrate metabolism enzymes and enzymes involved in formate production in *H. saccharophilum*, *A. fermentum*, and *A. tropicus* grown in the presence of glucose (nmol/(min mg protein))

Enzyme	Culture		
	H. saccharophilum	*A. fermentum*	*A. tropicus*
Hexokinase	354.0	900.0	871.0
Glucose-6-phosphate dehydrogenase	23.0	31.4	178.5
6-Phosphogluconate dehydrogenase	1.6	23.2	n.d.
2-Keto-3-deoxy-6-phosphogluconate aldolase	n.d.	55.7	17
6-Phosphogluconate dehydratase			
Phosphofructokinase	24.0	14.6	10.8
Glyceraldehyde-3-phosphate dehydrogenase	536.5	221.0	209
Fructose bisphosphate aldolase	166.6	356.0	141.6
Pyruvate-formate lyase	n.d.	–	0.28
Formate dehydrogenase	0.33	–	n.d.

Note: "n.d." stands for "not determined". "–" means that the determination was not performed.

extract in the medium and the elevation of cultivation temperature resulted in the involvement of 100% of the glucose in the glycolytic pathway [16]. If glutamate was present in the culture medium as a source of nitrogen, inactivation of the tricarboxylic acid cycle occurred and, as was shown for *B. thuringiensis*, this bacillus utilizes 5 to 16% of glucose via the HMP pathway [15, 16]. For *H. saccharophilum*, a concentration of yeast extract equal to 0.1 g/l is sufficient for growth. For *A. tropicus* and *A. fermentum*, an increase in the concentration of this substance leads to an increase in the growth rate. Since *H. saccharophilum*, *A. tropicus*, and *A. fermentum* obligately depend on yeast extract as the source of nitrogen for growth, we can say with sufficient certainty that, in the bacteria under study, as in *B. thuringiensis*, the main pathway is the FBP pathway, which may be involved in the catabolism not only of glucose but also of other substrates utilized by these bacteria. The enzymes of the other pathways of glucose metabolism might also be synthesized in the cells constitutively, but they may be involved in energy metabolism only under certain growth conditions.

The alkaliphilic bacilli cultivated in a medium with glucose can maintain the pH_{in} value at a level that is at least two units lower than the pH_{out} value [4]. Under microaerobic conditions, the obligate alkaliphile *A. tropicus*, which grows in a pH range from 8.5 to 11.5, can maintain the intracellular pH at a neutral and slightly alkaline level owing to one of the two known mechanisms: the active mechanism of pH_{in} maintenance operating during aerobic respiration [4] or the mechanism utilizing glycolysis energy [18]. The glucose-6-phosphate dehydrogenase of *A. tropicus* is active at the pH values of the reaction mixture from 6.0 to 10.2 with an optimum at pH 7.6–7.8. This pH range and optimal value correspond to the pH values that, according to calculations, can be maintained in the cells of this bacillus at pH_{out} values within its pH growth range [9]. However, the fructose bisphosphate aldolase of *A. tropicus* is active in a range of pH values (4.5 to 12.2) that is wider then the pH range that can be maintained inside the cell. Most of the data on the pH dependence of the enzymes of carbohydrate catabolism of alkaliphilic bacilli was obtained for the enzymes involved in polysaccharide decomposition; this is due to their industrial applications. All these enzymes are extremely tolerant of high pH values. Some of them, like the enzymes of *A. tropicus*, display the highest activity when the pH is close to neutral values [19]; others have an optimum at higher pH values [5].

Alkaliphilic bacteria differ from neutrophilic bacteria in that they do not form neutral catabolites such as acetone, acetoin, butanol, or ethanol [20]. However, the alkaliphilic bacilli and the haloanaerobe studied in this work form ethanol in considerable amounts. The main fermentation product of these bacteria is formate. *A. tropicus* is likely to form formate from pyruvate via the reaction catalyzed by pyruvate formate lyase. The conditions for determining the activity of this enzyme were not optimized; therefore, the activity value measured is low, constituting 0.28 mmol/(min mg protein). The presence of formate dehydrogenase in *H. saccharophilum* indicates that, in this bacterium, formate can be synthesized from CO_2. This process was discovered for the first time in *Clostridium thermoaceticum* [14], a bacterium that forms up to 30% of acetate from CO_2, which acts as the terminal acceptor of the electrons generated in the course of fermentation.

ACKNOWLEDGMENTS

We are grateful to G.A. Zavarzin and T.N. Zhilina for their interest in this work and helpful discussion of the results. The work was supported by the Russian Foundation for Basic Research, project nos. 01-04-06417 and 02-04-48286.

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