ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 3, pp. 273–279. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

Physiological and Biochemical Properties of the Alkaliphilic Anaerobic Hydrolytic Bacterium *Alkaliflexus imshenetskii*

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Received April 1, 2008

Abstract—The growth of *Alkaliflexus imshenetskii* and concentrations of metabolites produced by this microorganism during growth on various organic substrates were studied. It was shown that, although the composition and quantitative ratios of the fermentation products depended on the substrates utilized, acetate and succinate were always the major metabolites, while only minor amounts of formate were produced. During growth on xylan and starch, diauxy was observed caused by the successive decomposition of oligosaccharides and monosaccharides. It was demonstrated that, when grown on cellobiose, *A. imshenetskii* is capable of succinate fermentation mediated by phosphoenolpyruvate carboxykinase, pyruvate kinase, fumarate reductase, pyruvate ferredoxin oxidoreductase, malate dehydrogenase, and methylmalonyl-CoA decarboxylase. Succinate may be both the intermediate and final product of the *A. imshenetskii* metabolism, being fermented to propionate by methylmalonyl-CoA decarboxylase.

Key words: alkaliphiles, hydrolytic bacteria, cellobiose, succinate, succinate fermentation.

DOI: 10.1134/S0026261709030035

Alkaliflexus imshenetskii is an alkaliphilic hydrolytic anaerobic microorganism isolated from the low-mineralized alkaline Lake Verkhnee Beloe (Buryatia, Russia) [1]. According to the modern classification, *A. imshenetskii* belongs to the family *Marinilabiaceae*, order *Bacteroidales*. Capability for microcyst formation, carotenogenesis, and gliding motility are the characteristic traits of this microorganism. *A. imshenetskii* is capable of decomposing plant polymers (xylan and starch), as well as mono- and disaccharides. Among disaccharides, cellobiose is the most efficient energy substrate. The microorganism exhibits low cellulolytic activity. Growth occurs in a salinity range of 0.8–53 g/l Na^+ (with an optimum at 20 g/l) and in a pH range of 7.2–10.2 (with an optimum at pH 8.5).

It was previously demonstrated that *A. imshenetskii* is capable of mixed fermentation of sugars, producing succinate, acetate, and propionate (major metabolites), as well as formate, fumarate, and hydrogen (minor metabolites) [1]:

$$3C_{12}H_{22}O_{11} + 16OH^- \longrightarrow 4(CH_2)_2(COO^-)_2 + 4CH_3COO^- + 4CH_3CH_2COO^- + 17H_2O.$$

The closest relatives of *A. imshenetskii, Anaerophaga thermohalophila* and *Cytophaga fermentans*, use the same pathway [2].

Our further studies demonstrated that propionate does not always prevail among the fermentation products and may even be absent. In this case, succinate and acetate are the major metabolites. Hence, it is likely that *A. imshenetskii* is capable of succinate fermentation.

It is well known that succinate may be both the final and intermediate product of *A. imshenetskii* metabolism; it may be fermented to propionate [3] by propionyl-CoA transferase. For instance, in the case of the obligate anaerobe *Bacteroides fragilis*, succinate is converted to propionate through a series of successive stages [4], forming a "loop". At the first stage, propionyl-CoA : succinate CoA transferase catalyzes the transfer of succinate and propionyl-CoA into succinyl-CoA and propionate. Then, succinyl-CoA is transformed into methylmalonyl-CoA by methylmalonyl-CoA mutase. CO_2 is then cleaved from methylmalonyl-CoA by methylmalonyl-CoA decarboxylase, and the resulting propionyl-CoA interacts with succinate and is converted to propionate. In the anaerobe *Veillonella alcalescens* fermentation of succinate to propionate and CO_2 occurs via the same pathway [5, 6]. Using a cell extract of *Propionigenium modestum*, we demonstrated that succinate is converted to propionate and CO_2 via a series of successive stages that includes the production of succinyl-CoA, methylmalonyl-CoA, and propionyl-CoA. The latter reaction is mediated by the membrane-bound methylmalonyl-CoA decarboxylase which catalyzes exergonic decarboxylation with a Na^+ ion transport across the membrane [7].

The aim of the present work was to study the pathways of cellobiose fermentation to succinate in *A. imshenetskii*, as well as to determine the quantitative ratios of fermentation products obtained on various substrates

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and to assess the activities of the key enzymes of the *A. imshenetskii* metabolism.

MATERIALS AND METHODS

Organism and cultivation. In our study, we used the type strain *Alkaliflexus imshenetskii* Z-7010^T (=DSM 15055^T), obtained from T.N. Zhilina.

The bacteria were cultivated under strictly anaerobic conditions at 37°C on a mineral medium (pH 9.3) containing the following (g/l) [1]: KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.05; NH_4Cl, 0.2; Na_2CO_3, 7.4; $NaHCO_3$; 18.5; yeast extract, 0.2; $Na_2S \cdot 9H_2O$, 0.5, trace element solution (according to Lippert), 1 ml [8]; vitamin solution (according to Wolin, [9]), 2 ml; and 0.04% resazurin, 2 ml. The following substrates were used (%, wt/vol): cellobiose, 0.3; glucose, 0.3; oat spelt xylan, 0.2, and rice starch, 0.2.

Oxygen was removed from the medium by boiling and subsequent flushing the medium with N_2 gas for 15 min. The medium was cooled and supplemented with $NaHCO_3$, yeast extract, and a reducing agent. The reduced medium was dispensed into Hungate tubes or vials under nitrogen flow and sterilized in an autoclave at 121°C for 30 min. Mid-logarithmic phase cultures (1–3% vol/vol) were used as inocula. Cultivation on each substrate was performed in triplicate. Experimental points on the plots are an average of all measurements.

Determination of the growth rate. The growth rate of the cultures was assessed by an increase in the optical density at 600 nm in 1-cm cuvettes with a KFK-3 spectrophotometer (Russia). The growth rate of the cultures cultivated on the media containing insoluble starch or xylan was assessed by the total cell protein content determined by the Lowry method [10].

Analytical methods. The contents of fermentation products and sugars were determined by high-performance liquid chromatography on a Stayer chromatograph (Aquilon, Russia) equipped with an Aminex HPX-87H column (Bio-Rad, United States) and a refractometer detector. Elution was carried out at room temperature with 5 mM H_2SO_4. Intact cells and insoluble debris were removed from the samples of microbial suspension collected at various growth stages by centrifugation at 13500 *g* for 3 min. The supernatant was supplemented with 0.1 M of the $CuSO_4$ solution in 5 M H_2SO_4 to remove dissolved sulfide and acidify the medium to pH 2. After 1–2-h incubation (in order to let the CuS precipitate mature), the suspension was centrifuged under the same conditions. The supernatant was then injected into the chromatograph.

Obtaining of cell-free extracts. Bacterial cells were harvested by centrifugation at 9500 *g* for 15 min and resuspended in 6–8 ml of 50 mM HEPES buffer (pH 8.0) prepared using oxygen-free water and containing 0.1 mM EDTA, 10 mM sodium formate, and 25 mM sodium thioglycolate. The cell suspension was sonicated twice under aerobic conditions using a CPX130PB sonicator (Cole-Parmer, United States) at 0.4 mA for 0.5 min. To remove the cell debris, disrupted cells were centrifuged for 40 min at 4000 *g*. The supernatant was used for determination of the enzymatic activity.

Methods for determining the enzymatic activity. The enzymatic activities were determined by the oxidation or reduction of NADH or NADPH at 340 nm on a Specord recording spectrophotometer (Germany). In all cases, the volume of the reaction mixture (pH 8.5) was 1.05 ml. The enzymatic activity was expressed in nmol substrate/(min mg protein).

The compositions of the reaction mixtures used to determine the enzymatic activity were the following [11]:

Malate dehydrogenase (EC 1.1.1.37), 5 mM sodium malate; 1 mM NAD; 50 mM Tris–HCl buffer.

Pyruvate ferredoxin oxidoreductase (EC 1.2.7.1), 50 mM sodium pyruvate; 0.2 mM CoA; 25 mM dithiothreitol; 1 mM benzyl viologen; 50 mM Tris–HCl buffer; anaerobic conditions.

Fumarate reductase (EC 1.3.1.6), 5 mM sodium fumarate; 0.15 mM NADH; 50 mM Tris–HCl buffer.

Methylmalonyl-CoA decarboxylase (EC 4.1.1.41), 50 mM KCl; 0.15 mM methylmalonyl-CoA; 0.2 mM NADH; 50 mM Tris–HCl buffer.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.49), 10 mM $MgSO_4$; 10 mM ADP; 25 mM $NaHCO_3$; 20 mM phosphoenolpyruvate; 0.20 mM NADH; 2 U/mg malate dehydrogenase; 50 mM Tris–HCl buffer.

Pyruvate kinase (EC 2.7.1.40), 100 mM KCl; 10 mM $MgCl_2$; 0.16 mM NADH; 0.8 mM phosphoenolpyruvate; 2 mM ADP; 5 U/ml lactate dehydrogenase; 50 mM Tris–HCl buffer [12].

RESULTS AND DISCUSSION

Growth of *A. imshenetskii* on cellobiose. To determine the quantitative ratios of the products obtained in the course of cellobiose fermentation, bacteria were cultivated on this substrate. Figure 1 shows the growth dynamics. It was found that succinate and acetate were the major products of fermentation, and formate was a minor product. Pyruvate and fumarate (not shown on Fig. 1) were detected in trace quantities; they were probably present as intermediate products.

During the stationary growth phase, two molecules of succinate and one molecule of acetate were formed from one molecule of cellobiose. The substrate was completely utilized.

Growth of *A. imshenetskii* on glucose. In addition to the disaccharide cellobiose, *A. imshenetskii* is capable of fermenting monosaccharides, including glucose. Although cellobiose consists of glucose monomers, a different pattern was observed for strain Z-7010 grown on glucose (Fig. 2). In this case, metabolite ratios dif-

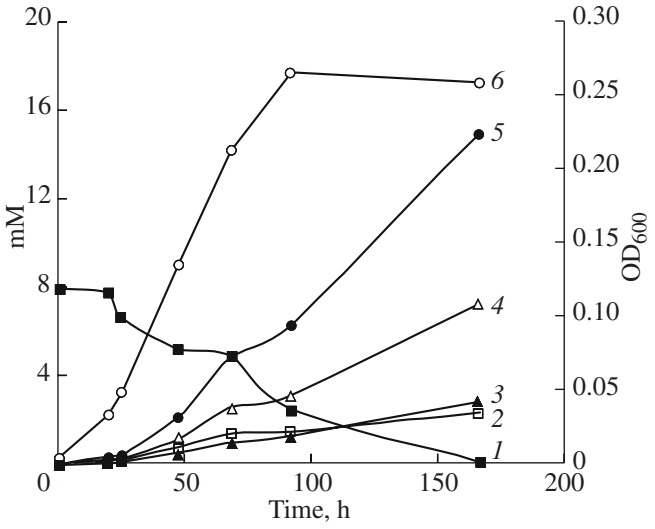

Fig. 1. Growth and products of the *A. imshenetskii* metabolism in cellobiose-containing medium: cellobiose (*1*); formate (*2*); pyruvate (*3*); acetate (*4*); succinate (*5*); OD_{600} (*6*).

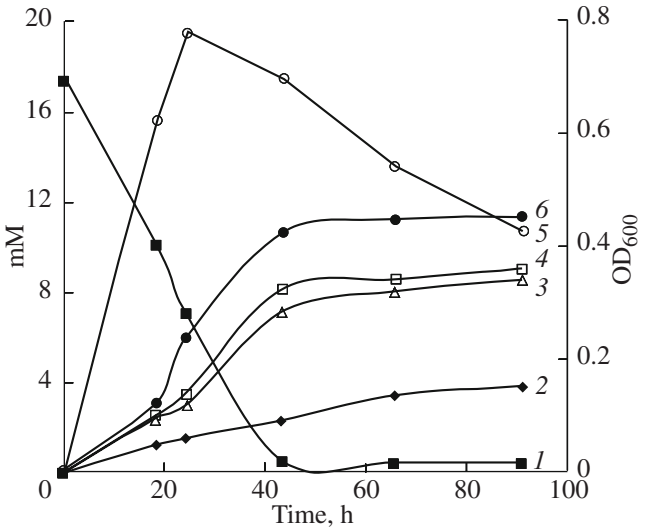

Fig. 2. Growth and products of the *A. imshenetskii* metabolism on glucose-containing medium: glucose (*1*); propionate (*2*); acetate (*3*); formate (*4*); OD_{600} (*5*); succinate (*6*).

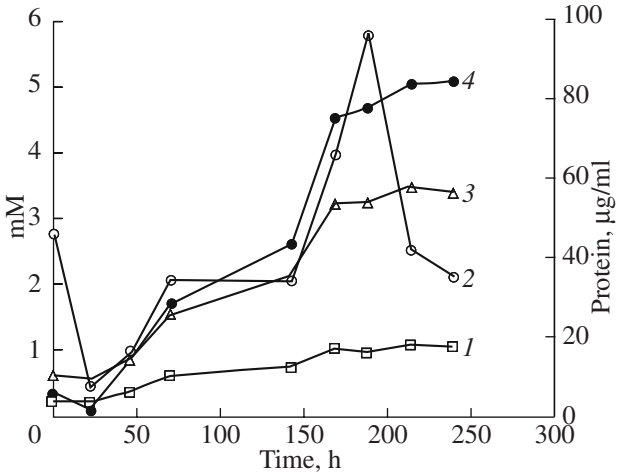

Fig. 3. Growth and products of the *A. imshenetskii* metabolism on xylan-containing medium: formate (*1*); cell protein (*2*); acetate (*3*);succinate (*4*).

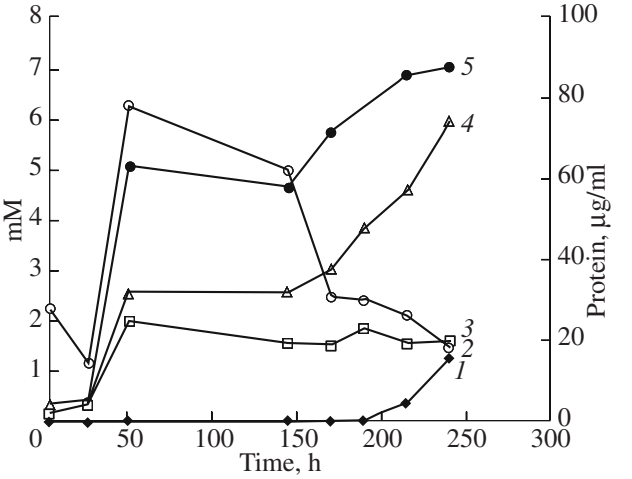

Fig. 4. Growth and products of the *A. imshenetskii* metabolism on starch-containing medium: propionate (*1*); formate (*2*); cell protein (*3*); acetate (*4*); succinate (*5*).

fered significantly from those observed during growth on cellobiose. In addition to succinate and acetate, formate was among the major metabolites; succinate and acetate were present in equal amounts. Besides, a large amount of propionate was present as well. Minor amounts of malate, pyruvate, and cellobiose were detected in the samples (not shown on Fig. 2).

Three molecules of succinate, two molecules of acetate, and two molecules of formate are produced from four molecules of glucose.

Growth of *A. imshenetskii* on polysaccharides. In alkaline lakes, the remains of shoreline vegetation and green algae may be the sources of polysaccharides that become available for biodegradation by the microbial communities of these lakes. Cellulose is the major plant polysaccharide; however, *A. imshenetskii* showed virtually no cellulolytic activity and grew only on starch and xylan [1].

Figure 3 shows the dynamics of *A. imshenetskii* growth on starch and xylan, and the dynamics of the metabolite production are shown in Fig. 4.

In contrast to growth on monosaccharides, diauxic (two-stage) growth was observed on xylan. Figure 3 shows that, within the first 24 h of cultivation, autolysis

Activities of the key enzymes of succinate fermentation in *A. imshenetskii* grown on cellobiose

Enzyme	Specific activity, nmol/(min mg protein)
Phosphoenolpyruvate carboxykinase	31.5
Pyruvate kinase	130.5
Fumarate reductase	121.2
Pyruvate ferredoxin oxidoreductase	157.8
Malate dehydrogenase	189.0
Methylmalonyl-CoA decarboxylase	126.6
Alcohol dehydrogenase	ND

Note: ND, not detected.

of the inoculum cells was observed, which can be attributed to the population variability of bacteria and elimination of the cells incapable of hydrolyzing xylan. Within 25–70 h of cultivation, the total amounts of cell protein and metabolites increased due to utilization of oligosaccharide hydrolysis products. Within the next 80 h of cultivation, the first stationary phase was observed; it is probably due to induction of the enzymes involved in monosaccharide utilization. Then, a new increase in the amounts of cell protein and metabolites was observed indicating that the monosaccharides released by xylan hydrolysis were utilized.

During growth on starch, the dynamics of metabolite production was also unusual. Figure 4 shows that, after a brief lag phase (24 h) which, as in the case of xylan, was accompanied by the inoculum autolysis, rapid growth was observed. The onset of the stationary growth phase was detected after 50-h incubation; the duration of this phase was over 100 h, unlike growth on xylan when the stationary phase was almost absent. After 150-h incubation, metabolites continued to accumulate, although the amount of cell protein decreased. This can be attributed to the metabolic activity of the remaining viable stationary-phase cells. Judging from the duration of the period of metabolite detection (accumulation) in the cultures grown on polymeric substrates (as compared to growth on monosaccharides), prolonged and stable functioning of extracellular hydrolases providing monomers as carbon sources may be suggested.

In both cases, succinate, acetate, and formate (metabolites typical of *A. imshenetskii*) were formed in a ratio of approximately 3 : 2 : 1. The ratio between the final products is similar to that determined for the culture grown on glucose. Moreover, propionate was produced by *A. imshenetskii* cultures grown on starch (Fig. 4). Minor amounts of pyruvate and malate were detected.

Key enzymes of the *A. imshenetskii* metabolism. It is well known that succinate is a usual intermediate or final metabolite of various anaerobic microorganisms [13–15]. These organisms are usually capable of mixed fermentation, i.e., in addition to succinate, they may produce other metabolites, including acetate, lactate, formate, ethanol, and propionate. The ratios of these metabolites for different microorganisms may vary significantly. Figure 5 shows a schematic diagram for succinate fermentation, the so-called phosphoenolpyruvate carboxykinase pathway.

Although in the majority of succinate-producing microorganisms, the phosphoenolpyruvate carboxykinase pathway prevails, some microorganisms are capable of converting phosphoenolpyruvate to oxaloacetate by both phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase (phosphoenolpyruvate carboxylase pathway) [16]. The main difference of the phosphoenolpyruvate carboxylase pathway is that oxaloacetate is converted to aspartate (not to malate), which in turn is converted to fumarate.

Since *A. imshenetskii* grown on various sugars produces succinate, acetate, and formate as the major metabolites, it was suggested that this microorganism carries out succinate fermentation according to the scheme shown in Fig. 5.

To verify this hypothesis, we measured the activities of the key enzymes involved in different stages of this pathway. When grown on cellobiose, activity of phosphoenolpyruvate carboxykinase, pyruvate kinase, fumarate reductase, pyruvate ferredoxin oxidoreductase, malate dehydrogenase, and methylmalonyl-CoA decarboxylase were revealed. Their specific activities are listed in the table. No alcohol dehydrogenase activity was detected.

On the basis of the data obtained, a scheme for cellobiose catabolism in *A. imshenetskii* was suggested (Fig. 6). It demonstrates that in *A. imshenetskii*, the branches leading to lactate and ethanol production were missing. High methylmalonyl-CoA decarboxylase activity indicates that the pathway involves conversion of succinate to propionate via formation of methylmalonyl-CoA. Despite the relatively high activity of this enzyme, propionate is not always the final product; therefore, this process may be reversible.

To elucidate whether the succinate–propionate cycle similar to the one found in *Bacteroides fragilis* [4] is active in *A. imshenetskii*, we incubated the suspension of *A. imshenetskii* cells in the presence of succinate or propionate. The suspension was incubated in carbonate–bicarbonate buffer (pH 9.3) at 35°C for 6 h. The suspension was sampled every 40 min.

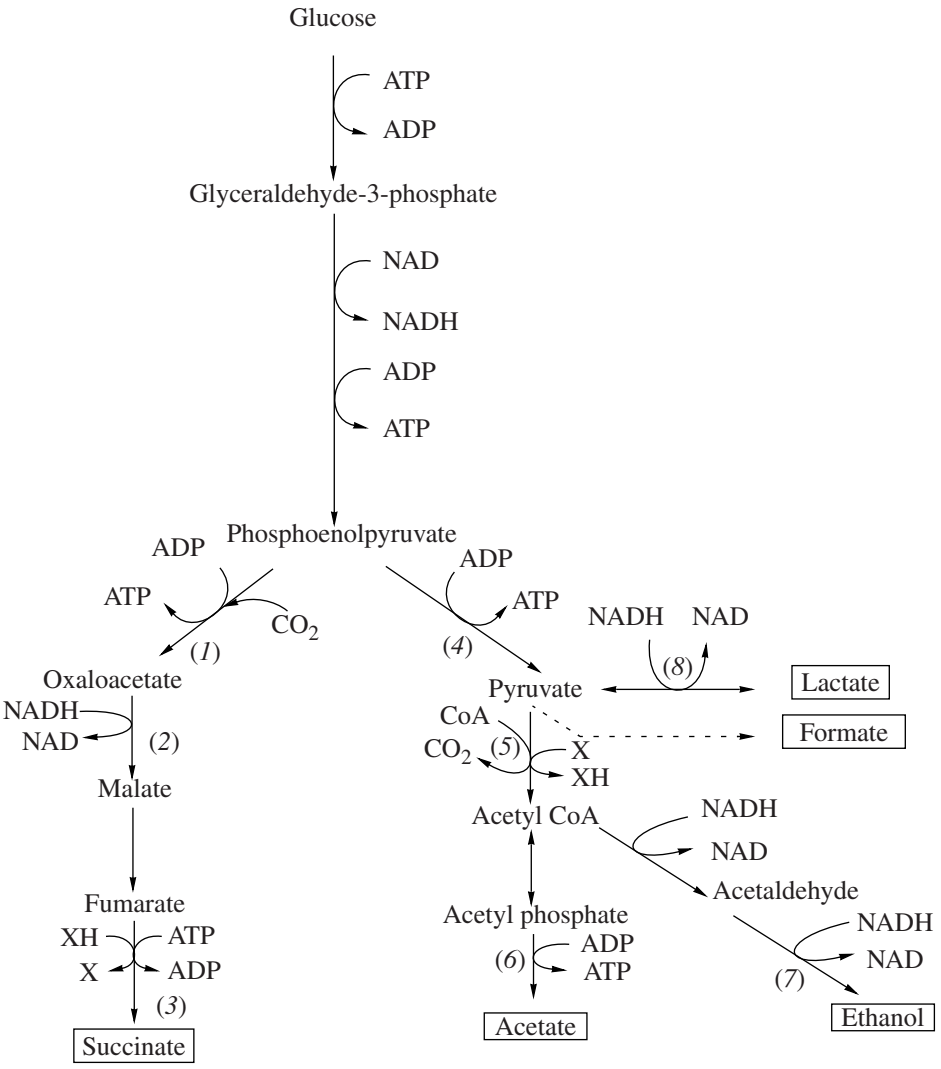

Fig. 5. General scheme of succinate fermentation according to [17]: phosphoenolpyruvate carboxykinase (*1*); malate dehydrogenase (*2*); fumarate reductase (*3*); pyruvate kinase (*4*); pyruvate ferredoxin oxidoreductase (*5*); acetate kinase (*6*); alcohol dehydrogenase (*7*); lactate dehydrogenase (*8*).

In the course of incubation of the cell suspension in the presence of propionate, production of succinate was observed; small amounts of acetate and formate were produced as well. Figure 7 shows that metabolite accumulation was not accompanied by significant consumption of the substrate. Therefore, it may be suggested that the succinate–propionate cycle is active in *A. imshenetskii*. Propionate involved in this cycle is initially consumed in the course of *A. imshenetskii* metabolism, and then it is synthesized from succinate by methylmalonyl-CoA decarboxylase.

A different pattern was observed during incubation on succinate (Fig. 8). In this case, only acetate and not propionate was produced. The reactions on the phosphoenolpyruvate–carboxykinase route (Fig. 6) are reversible. On the route from succinate to acetate via phosphoenolpyruvate, three molecules of ATP are formed at the level of substrate phosphorylation; only one of these molecules is consumed. The synthesis of propionate from succinate is not accompanied by the ATP synthesis at the level of substrate phosphorylation. On this route, oxidative phosphorylation occurs, which results in formation of only one ATP molecule [7]. This is probably the reason why *A. imshenetskii* uses the most efficient energy pathway.

ACKNOWLEDGMENTS

The authors thank Prof. G.A. Zavarzin for valuable advice and participation in discussing the results.

This work was supported by the "Leading Scientific Schools" Program of the President of the Russian Fed-

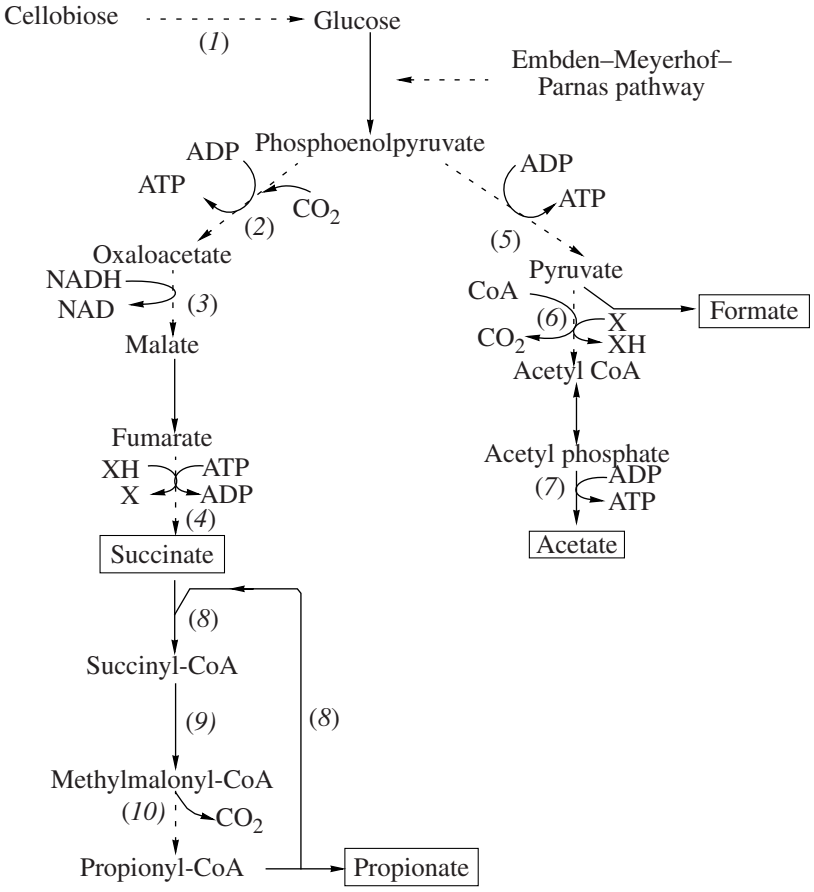

Fig. 6. Proposed scheme of cellobiose catabolism in *A. imshenetskii.* The dotted lines indicate the reactions and enzymes detected in *A. imshenetskii.* cellobiose + phosphorylase (*1*); phosphoenolpyruvate carboxykinase (*2*); malate dehydrogenase (*3*); fumarate reductase (*4*); pyruvate kinase (*5*); pyruvate ferredoxin oxidoreductase (*6*); acetate kinase (*7*); succinate propionyl-CoA : CoA-transferase (*8*); methylmalonyl-CoA mutase (*9*); methylmalonyl-CoA decarboxylase (*10*).

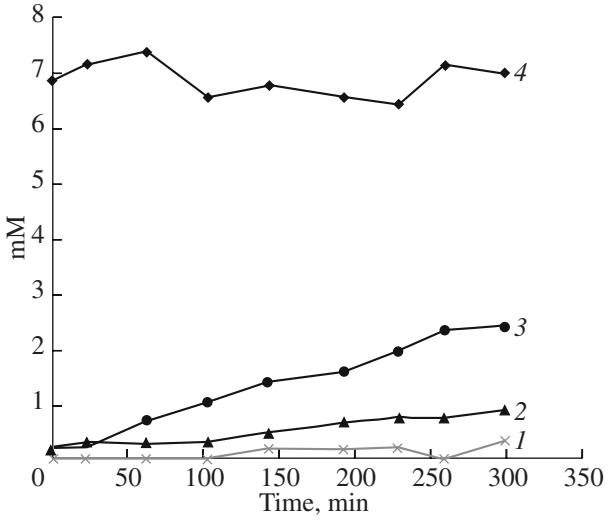

Fig. 7. Incubation of the *A. imshenetskii* cell suspension in the presence of propionate: formate (*1*); acetate (*2*); succinate (*3*); propionate (*4*).

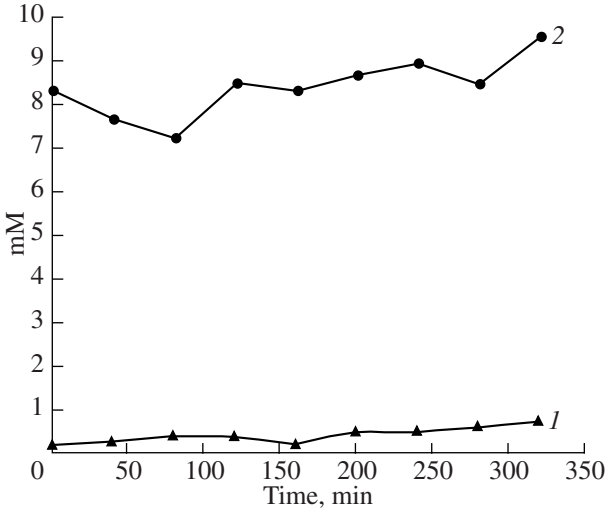

Fig. 8. Incubation of the cell suspension of *A. imshenetskii* in the presence of succinate: acetate (*1*); succinate (*2*).

eration (grant no. NSh-1221.2008.4) and the "Molecular and Cell Biology" Program of the Presidium of the Russian Academy of Sciences (G.A. Zavarzin).

REFERENCES

1. Zhilina, T.N., Appel, R., Probian, C., Brossa, E.L., Harder, J., Widdel, F., and Zavarzin, G.A., *Alkaliflexus imshenetskii* gen. nov.,sp. nov. a New Alkaliphilic Gliding Carbohydrate-Fermenting Bacterium with Propionate Formation from a Soda Lake, *Arch. Microbiol.,* 2004, vol. 182, no. 2–3, pp. 244–253.
2. Denger, K., Warhmann, R., Ludwig, W., and Schink, B., *Anaerofaga thermohalophila* gen. nov., sp. nov., a Moderately Thermohalophilic, Strictly Anaerobic Fermentative Bacterium, *Int. J. Syst. Evol. Microbiol.,* 2002, vol. 52, pp. 173–178.
3. Scheifinger, C.C. and Wolin, M.J., Propionate Formation from Cellulose and Soluble Sugars by Combined Cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium, Appl. Microbiol.*, 1973, vol. 26, pp. 789–795.
4. Macy, J.M., Ljungdahl, L.G., and Gottschalk, G., Pathway of Succinate and Propionate Formation in *Bacteroides fragilis, J. Bacteriol.*, 1978, vol. 134, pp. 84–91.
5. De Vries, W., Rietveld-Struijk, R.M., and Stouthamer, A.H., ATP Formation Associated with Fumarate and Nitrate Reduction in Growing Cultures of *Veillonella* alcalescens, *Antonie Van Leeuwenhoek. J. Microbiol. Serol.*, 1977, vol. 43, pp. 153–167.
6. Hilpert, W. and Dimroth, P., Conversion of the Chemical Energy of Methylmalonyl-CoA Decarboxylation into a Na^+ Gradient, *Nature*, 1982, vol. 296, pp. 584–585.
7. Hilpert, W., Schink, B., and Dimroth, P., Life by a New Decarboxylation-Depent Energy Conversion with Na^+ as Coupling Ion, *EMBO J.*, 1984, vol. 3, pp. 1665–1670.
8. Pfenning, N. and Lippert, K.D., Über Das Vitamin B-Bedürfnis Phototropher Schwefelbakterien, *Arch. Microbiol.*, 1966, vol. 55, pp. 245–246.
9. Wolin, E.A., Wolin, M.J., and Wolfe, R.S., Formation of Methane by Bacterial Extracts, *J. Biol. Chem.*, 1963, vol. 238, pp. 2882–2886.
10. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Rendall, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
11. Melville, S.B., Michel, T.A., and Macy, J.M., Pathway and Sites for Energy in the Metabolism of Glucose by *Selenomonas ruminantium, J. Bacteriol.,* 1988, vol. 170, pp. 5298–5304.
12. Cardenas, J., Dyson, R., and Strandholm, J., Bovine Pyruvate Kinases. Purification and Characterization of the Skeletal Muscle Isozyme, *J. Biol. Chem.*, 1973, vol. 248, p. 6931.
13. Zeikus, J.G., Chemical and Fuel Production by Anaerobic Bacteria, *Annu. Rev. Microbiol.,* 1980, vol. 34, pp. 423–464.
14. Zeikus, J.G., Jain, M.K., and Elankovan, P., Biotechnology of Succinic Acid Production and Markets for Derived Industrial Products, *Appl. Microbiol. Biotechnol.,* 1999, vol. 51, pp. 545–552.
15. Lee, P.C., Lee, W.G., Kwon, S., Lee, S.Y., and Chang, H.N., Succinic Acid Production by *Anaerobiospirillum succiniciproducens*: Effects of the H_2/CO_2 Supply and Glucose Concentration, *Enzyme Microb. Technol.*, 1999, vol. 24, pp. 549–554.
16. Van der Welf, M.J., Guettler, M.V., Jain, M.K., and Zeikus, J.G., Environmental and Physiological Factors Affecting the Succinate Product Ratio During Carbohydrate Fermentation by *Actinobacillus* sp. 130Z, *Arch. Microbiol.*, 1997, vol. 167, pp. 332–342.
17. Samuelov N.S., Lamed R., Lowe S., Zeikus J.G. Influence of CO_2-HCO_3^- Levels and pH on Growth, Succinate Production, and Enzyme Activities of *Anaerobiospirillum succiniciproducens, Appl. Environ. Microbiol.*, 1991, vol. 57, pp. 3013-3019.