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EXPERIMENTAL ARTICLES

Different Ionic Specificities of ATP Synthesis in Extremely Alkaliphilic Sulfate-Reducing and Acetogenic Bacteria

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Abstract—Ionic specificity of oxidative phosphorylation was studied in *Natroniella acetigena* and *Desulfonatronum lacustre*, which are new alkaliphilic anaerobes that were isolated from soda lakes and have a pH growth optimum of 9.5–9.7. The ability of their cells to synthesize ATP in response to the imposition of artificial ΔpH^+ and ΔpNa^+ gradients was studied. As distinct from other marine and freshwater sulfate reducers and extremely alkaliphilic anaerobes, *D. lacustre* uses a Na^+-translocating ATPase for ATP synthesis. The alkaliphilic acetogen *N. acetigena*, which develops at a much higher Na^+ concentration in the medium, generated primary ΔpH^+ for ATP synthesis. Thus, the high Na^+ concentrations and alkaline pH values typical of soda lakes do not predetermine the type of bioenergetics of their inhabitants.

Key words: alkaliphiles, anaerobes, homoacetogens, sulfate reducers, Na^+-ATP synthase, H^+-ATP synthase, bioenergetics.

Extremely halophilic and alkaliphilic bacteria are of considerable interest in regards to their energy metabolism. At high pH values and high Na^+ concentrations, alkaliphiles have to solve two problems. They are to maintain intracellular pH at a level below pH 8, and they are to perform transmembrane transport of the ions necessary for their energy metabolism. In most bacteria, ATP synthesis involves an H^+-translocating ATPase; however, alkaliphilic bacteria and archaea, growing under conditions of proton deficiency and at high Na^+ concentrations, have to use special mechanisms of ATP synthesis, including the Na^+-translocating ATPase [1]. A primary sodium cycle has been found in a marine alkalitolerant bacterium *Vibrio alginolyticus*: as a result of the operation of a sodium pump, a primary sodium gradient is built up, which is then used by a Na^+-translocating ATPase for ATP synthesis [2]. On the other hand, aerobic extremely alkaliphilic bacilli growing at pH 11 employ a primary proton cycle. The pH value inside the cells of these bacilli is 3 units lower than in the medium; however, ATP synthesis occurs due to the operation of an H^+-translocating ATPase [3].

Among neutrophilic prokaryotes, there are some organisms, e.g., secondary anaerobes (acetogens and methanogens), that use sodium gradient for ATP synthesis. In the acetogenic bacterium *Acetobacterium woodii*, the physiological role of the Na^+-translocating ATPase is to couple the Wood–Ljungdahl pathway, whose operation produces a sodium gradient, with the synthesis of ATP [4]. In the methanogen *Methanosarcina mazei* Gö I, the primary sodium gradient is formed as a result of a methyltransferase reaction and is used for ATP synthesis. In addition to the Na^+-translocating ATPase of the F_1F_0 type, methanogens were also found to possess an H^+-translocating ATPase of the A_1A_0 type [5].

From continental alkaline lakes of central Africa and the Transbaikal Region, extremely alkaliphilic anaerobes with a growth optimum at pH 9.5–9.7 were isolated. Among them, most interesting to us were the extremely haloalkaliphilic bacterium *Natroniella acetigena*, which grows on media with ethanol or lactate at a NaCl concentration of 1.57% (total Na^+ concentration, 2 M) and pH 9.7 [6], and the extremely alkaliphilic bacterium *Desulfonatronum lacustre* which reduces sulfate at the expense of H_2, ethanol, or formate and grows at pH 9.5 in the absence of NaCl at a total Na^+ concentration of 0.2 M [7].

The question is how the energy metabolism of alkaliphilic anaerobes proceeds at extremely high Na^+ concentrations and alkaline pH values (i.e., at low concentrations of protons in the medium). In our previous works, we showed that the energy metabolism of the alkaliphilic bacteria *Natroniella acetigena* and *D. lacustre* relies, regardless of the substrate utilized, on oxidative phosphorylation that involves membrane ATPase [8, 9]. In *D. lacustre*, a proton cycle functions, which involves periplasmic and intracellular hydroge-

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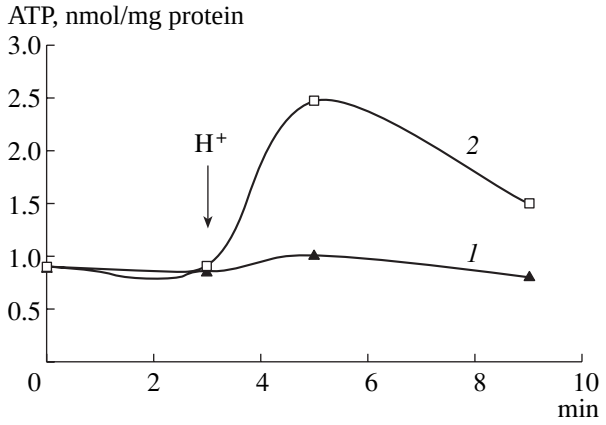

Fig. 1. Synthesis of ATP by *N. acetigena* cells washed and suspended in substrate-free medium (*1*) in control variant and (*2*) in response to the imposition of a proton gradient (the arrow indicates the moment of HCl addition).

nases and is coupled to electron transport that occurs during the oxidation of hydrogen or organic substrates in the course of sulfate reduction [9]. Inhibitory analysis of the energy metabolism of the alkaliphiles under consideration showed the operation of both the proton and sodium cycles.

The aim of the present work was to establish the nature of the ion necessary for ATP synthesis by the membrane ATPases of the extremely haloalkaliphilic homoacetogenic bacterium *N. acetigena* and the alkaliphilic sulfate-reducing bacterium *D. lacustre.*

MATERIALS AND METHODS

Organisms and cultivation conditions. This work used the type strains *N. acetigena* Z-7937^T (=DSM 9952) and *D. lacustre* Z-7951^T (=DSM 10312) obtained from T.N. Zhilina. *N. acetigena* that was grown at 37°C under strictly anaerobic conditions on mineral medium with pH 9.7 containing ethanol (0.5%) and the following components (g/l): KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 1.0; KCl, 0.2; NaCl, 15.7; Na_2CO_3, 68.3; $NaHCO_3$, 38.3; yeast extract, 0.2; $Na_2S \cdot 9H_2O$, 1.0; Lippert's trace element solution, 1 ml; Wolin's vitamin solution, 2 ml; 0.04% resazurin solution, 2 ml [6]. *D. lacustre* was grown at 37°C under strictly anaerobic conditions on mineral medium with pH 9.5 containing the following components (g/l): K_2HPO_4, 0.2; KCl, 0.2; $MgCl_2 \cdot 6H_2O$, 0,1; NH_4Cl, 1,0; Na_2SO_4, 3.0; Na_2CO_3, 2.76; $NaHCO_3$, 10.0; yeast extract, 1.0; $Na_2S \cdot 9H_2O$, 0.5; Whitman's trace element solution (1 ml); Wolin's vitamin solution (2 ml); 0.04% resazurin solution, 2 ml [7]; formate was added as the electron donor. To create anaerobic conditions, the media were boiled and flushed with 100% N_2.

The inoculum was a log-phase culture taken in an amount of 3 vol %.

Obtaining of cell suspensions. Late-log-phase cultures were centrifuged under anaerobic conditions for 1 h at 4600 g and 4°C. Cells were washed two times and resuspended (0.8–1.2 mg protein/ml) either in cultivation medium or in deoxygenated 50 mM Tris buffer (pH 9.5) containing 10 mM $MgCl_2$ and 10 mM KCl.

Determination of intracellular ATP content. A freshly prepared cell suspension was incubated for 30 min at 37°C under a nitrogen atmosphere in the presence or absence of ionophores and substrates. ATP synthesis was induced by the addition of 6 N HCl in an amount that shifted the pH value by 2 units. Artificial ΔpNa^+ was imposed by the addition of 200 mM NaCl to cells incubated in medium or buffer devoid of sodium. Intracellular ATP was extracted with 2 M per-

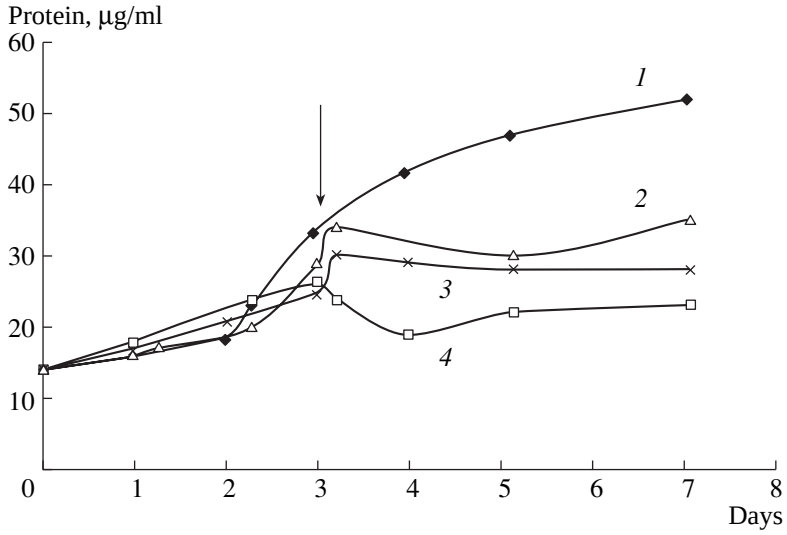

Fig. 2. Effect of (*2*) 50 μM monensin, (*3*) 500 μM DCCD, and (*4*) 100 μM FCCP on the growth of *D. lacustre* in formate-containing medium (the arrow indicates the moment of DCCD or ionophore addition). Curve *1* shows the growth of the control culture.

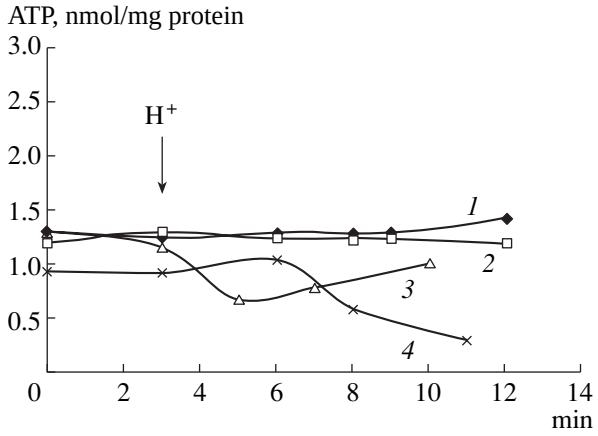

Fig. 3. Effect of proton gradient on ATP synthesis by *D. lacustre*: (*1*) control (unimpacted) cells; (*2*) cells washed and suspended in medium with sulfate and formate; (*3*) cells washed and suspended in medium devoid of sulfate and formate; (*4*) cells washed and suspended in Tris buffer (pH 9.5) containing 10 mM KCl and 10 mM $MgCl_2$. The arrow shows the moment of HCl addition to the experimental variants.

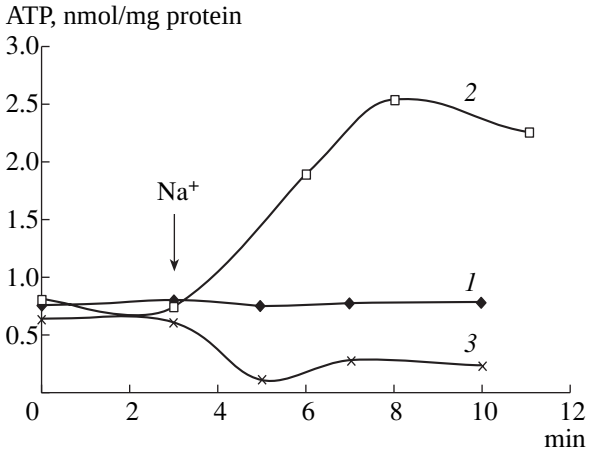

Fig. 4. Synthesis of ATP by *D. lacustre* cells washed and suspended in Tris buffer (pH 9.5) containing 10 mM KCl and 10 mM $MgCl_2$ (*1*) in control variant and in response to the imposition of a sodium gradient (the arrow indicates the moment of the addition of 0.2 M NaCl) (*2*) in the absence of monensin and (*3*) in the presence of 100 μM monensin.

chloric acid on ice for 30 min, followed by neutralization with 3 M KOH. To stabilize the pH value of the extract, Tris–HCl buffer (pH 7.4) containing 4 mM $MgCl_2$ was added. The ATP concentration was determined by the luciferin–luciferase method using the Immolum and Microlum luminescent ATP reagents manufactured at the Department of Chemical Enzymology, Moscow State University [10]. The luminescence intensity was measured on a RackBeta 1219 LKB–Wallac scintillation counter [11].

The biomass was determined as the cellular protein after sedimenting cells by centrifugation at 15 000 g for 3 min followed by cell hydrolysis in 1 N NaOH. The protein was determined by the Lowry method.

The inhibitor *N,N*-dicyclohexylcarbodiimide (DCCD) and the ionophores monensin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) purchased from Serva (Germany) were added to the growth medium or cell suspensions as ethanol solutions. Control cultures and cell suspensions were supplemented with corresponding amounts of ethanol (no more that 0.1%).

RESULTS AND DISCUSSION

Homoacetogenic bacteria can form acetate from various substrates (sugars, aromatic compounds, organic acids, alcohols, H_2/CO_2) and couple this reaction with Na^+- or H^+-dependent ATP synthesis; the mechanism of coupling involves the electron-transport chain or the direct transmembrane transport of ions. With respect to their bioenergetics, homoacetogenic bacteria can be divided into two groups. Members of the first group (*Clostridium thermoaceticum*) generate proton gradient (used for ATP synthesis by an H^+-translocating ATPase) via the operation of an electron-transport chain containing cytochrome *b* [12]. Homoacetogens of the second group (*Acetobacterium woodii* and *Thermoanaerobacter kivui*) do not contain cytochromes and generate primary sodium gradient in the course of methyltransferase reaction; this gradient is used by a Na^+-translocating ATPase [5].

To reveal an H^+-translocating ATPase in *N. acetigena*, we studied the ability of this bacterium to synthesize ATP in response to the imposition of an artificial proton gradient (ΔpH) on starving cells suspended in growth medium (pH 9.7) devoid of the carbon source. As can be seen from Fig. 1, a decrease in the pH value by 2 units resulted in a 2.5-fold increase in the intracellular ATP content (which reached 2.5 nmol/mg protein), indicating H^+-dependent ATP synthesis by *N. acetigena*. This result is in agreement with our earlier obtained data [8, 13] on the complete inhibition of growth and the acetogenesis of *N. acetigena* in media with ethanol or lactate at pH 9.7 by protonophores and Rhodamine G, which is known to affect the H^+-translocating ATPase.

ATP synthesis by *N. acetigena* was studied in cells suspended in mineral medium, since in buffers devoid of Na^+, ions cells undergo lysis. Therefore, it was impossible to investigate the effect of an artificial sodium gradient (ΔpNa^+). *N. acetigena* cells cannot withstand washing and suspension in sucrose-containing buffers or in buffers based on the mineral components of the cultivation medium but containing potassium instead of sodium. Earlier, we showed that the addition of monensin, protonophores, the inhibitor of the F_1F_0-type ATPase DCCD, or the inhibitor of H^+-translocating ATPases Rhodamine G also causes the rapid lysis of *N. acetigena* cells [8, 13]. Cell lysis in the

absence of Na^+ or upon the addition of inhibitors and ionophores suggests that the physiology of *N. acetigena* is based on a combination of the proton and sodium cycles and is adjusted to the considerable energy expenditures needed for the maintenance of cell integrity in the highly mineralized alkaline medium.

The energy metabolism of another group of secondary anaerobes, hydrogenotrophic sulfate-reducing bacteria, is based on the intracellular hydrogen cycle. The proton gradient formed due to the operation of the electron-transport chain is used for ATP synthesis, the transport of nutrients (including sulfate), and flagellum motion [14].

Figure 2 presents data on the effect of the F_1F_0 ATPase inhibitor DCCD and ionophores on the growth on *D. lacustre* in medium with formate. The growth was completely inhibited by the addition of 500 μM DCCD in the 72nd hour of growth (curve *3*). An analogous effect was produced by 50 μM monensin, which abolishes ΔpH and ΔpNa gradients on the membrane (curve *2*), and by 100 μM protonophore FCCP (curve *4*). The complete inhibition of growth by DCCD and the protonophore suggests that formate oxidation in the course of sulfate reduction is coupled to ATP generation via the chemiosmotic mechanism. On the other hand, the inhibitory effect of monensin on *D. lacustre* indicates that, during growth on formate, the bacterium requires sodium potential, which is established via Na^+/H^+ antiport. Thus, the energy metabolism of *D. lacustre* involves both proton and sodium cycles.

The capacity of *D. lacustre* for proton-dependent ATP synthesis was studied by imposing an artificial ΔpH on cells. As can be seen from Fig. 3, the ATP level in cell suspensions of *D. lacustre*, as distinct from that in cell suspensions of *N. acetigena*, did not respond to a decrease in pH either during incubation in cultivation medium (curve *2*) or medium devoid of organic substrate and sulfate (curve *3*) or in Tris buffer (pH 9.5) containing 10 mM $MgCl_2$ and 10 mM KCl (curve *4*). In the latter case, a slight decrease in the cellular ATP level was observed. These data demonstrate that *D. lacustre*, as distinct from other sulfate-reducing bacteria studied in this respect, does not use the proton gradient for ATP synthesis.

To elucidate the ability of *D. lacustre* cells to synthesize ATP at the expense of ΔpNa^+, twice-washed cells were suspended in 50 mM Tris buffer (pH 9.5) containing 10 mM $MgCl_2$ and 10 mM KCl. As distinct from *N. acetigena* cells, the cells of *D. lacustre* do not lyse in a sodium-free buffer. The addition of 200 mM NaCl after a 35-min incubation in a sodium-free buffer resulted in a 3.3-fold increase in the cellular ATP content (Fig. 4, curve *2*). When a buffer with pH 10.2 was used, ATP synthesis was not observed, which was probably due to the impairment of cell pH homeostasis and ATPase suppression. ATP synthesis in *D. lacustre* cells was inhibited by monensin (curve *3*), which abolishes ΔpNa and ΔpH.

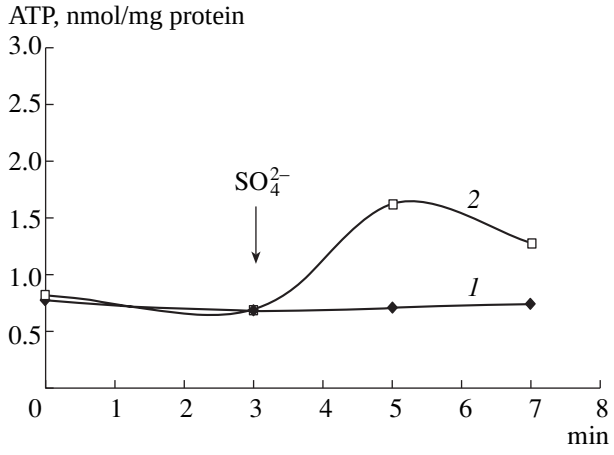

Fig. 5. Synthesis of ATP by *D. lacustre* cells washed and suspended in Tris buffer (pH 9.5) containing 10 mM KCl, 10 mM $MgCl_2$, and 80 mM formate (*1*) in control variant and (*2*) in response to the addition of 20 mM potassium sulfate (arrow).

In washed cells of *D. lacustre*, we also found sulfate-dependent ATP synthesis. The addition of 20 mM of potassium sulfate to cells that were incubated in Tris buffer supplemented with formate resulted in a twofold increase in the intracellular ATP content (Fig. 5). Thus, the oxidation of formate with sulfate by *D. lacustre* is coupled to energy generation.

In earlier studied sulfate-reducing bacteria, both those that couple sulfate reduction to the oxidation of organic substrates and those that couple it to the oxidation of hydrogen, the proton cycle played the key role in energy generation [14]. In all sulfate-reducing bacteria earlier studied in this respect, the H^+-translocating ATPase is involved in ATP synthesis [15].

The extremely alkaliphilic sulfate-reducing bacterium *D. lacustre* as distinct from the earlier-studied marine and freshwater sulfate-reducers and extremely alkaliphilic aerobic bacilli, relies on a Na^+-translocating ATPase for ATP synthesis. *D. lacustre* cells can generate proton gradient via the operation of the periplasmic and intracellular hydrogenases, as well as via the operation of the electron-transport chain, which contains cytochrome *c* [9]. The interconversion of the ΔpNa and ΔpH gradients may occur via Na^+/H^+ antiport, as it is the case with *Desulfovibrio salexigenes* [15]. It is believed that, under alkaline conditions, the electrochemical proton potential that is built up in the course of the operation of the electron-transport chain is used by the Na^+/H^+ antiporter to obtain the sodium transmembrane potential, which is the driving force of ATP synthesis. In extremely alkaliphilic bacteria, the Na^+/H^+ antiport plays the key role in the maintenance of pH homeostasis [3]. Paradoxically, *N. acetigena*, the other anaerobic alkaliphilic soda-lake isolate studied in the present work and growing at a much higher Na^+ concentration in the medium (2 M, pH 9.5), was shown

by inhibitory analysis to rely on the H^+-translocating ATPase for ATP synthesis. The presence of the H^+-translocating ATPase in *N. acetigena* cells cannot be excluded and requires experimental testing, which is, however, problematic in a Na^+-dependent organism.

The mechanism of proton-dependent ATP synthesis at high pH values remains obscure; it has been suggested that this mechanism may be nonchemiosmotic or may be characterized by a high H^+/ATP ratio [3].

As follows from our data, the high Na^+ concentrations and alkaline pH values typical of soda lakes do not predetermine the type of bioenergetics of their inhabitants. New alkaliphilic anaerobic isolates from soda lakes exhibit a wide diversity of energy generation mechanisms: acetogenesis, hydrogenotrophic sulfidogenesis, fermentation of sugars and amino acids, phototrophy, etc. [16]. Therefore, various combinations of the proton and sodium energy cycles can be anticipated to occur in these isolates.

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REFERENCES

1. Speelman, G., Poolman, B., and Konings, W.N., Na^+ as Coupling Ion in Energy Transduction in Extremophilic Bacteria and Archaea, *World J. Microbiol. Biotechnol.*, 1995, vol. 11, no. 1, pp. 58–70.
2. Skulachev, V.P., Bacterial Energetics at High pH: What Happens to the H^+ Cycle When the H^+ Concentration Decreases?, *Novartis Found Symp.*, 1999, vol. 221, pp. 200–213, discussion 213–217.
3. Krulwich, T.A., Hicks, D.B., Seto-Young, D., and Guffanti, A.A., The Bioenergetics of Alkaliphilic Bacilli, *CRC Crit. Rev. Microbiol.*, 1988, vol. 16, pp. 15–36.
4. Heinse, R. and Reidlinger, J., Müller, G., and Gottschalk, V., A Sodium-Stimulated ATP Synthase in the Acetogenic Bacterium *Acetobacterium woodii*, *FEBS*, 1991, vol. 295, no. 1/3, pp. 119–122.
5. Müller, G. and Gottschalk, V., The Sodium Ion Cycle in Acetogenic and Methanogenic Bacteria: Generation and Utilization of Primary Electrochemical Ion Gradient, *Acetogenesis*, Drake, H.L., Ed., New York: Chapman & Hall, 1994, pp. 127–156.
6. Zhilina, T.N., Zavarzin, G.A., Detkova, E.N., and Rainey, F.A., *Natroniella acetigena* gen. nov., sp. nov., an Extremely Haloalkalophilic, Homoacetic Bacterium: A New Member of *Haloanaerobiales*, *Curr. Microbiol.*, 1996, vol. 32, pp. 320–326.
7. Pikuta, E.V., Zhilina, T.N., Zavarzin, G.A., Kostrikina, N.A., Osipov, G.A., and Rainey, F.A., *Desulfonatronovibrio lacustre* gen. nov., sp. nov.: A New Alkaliphilic Sulfate-Reducing Bacterium Utilizing Ethanol, *Mikrobiologiya*, 1998, vol. 67, no. 1, pp. 127–135.
8. Pusheva, M.A., Pitryuk, A.V., Detkova, E.N., and Zavarzin, G.A., Bioenergetics of Acetogenesis in the Extremely Alkaliphilic Homoacetic Bacteria *Natroniella acetigena* and *Natronoincola histidinovorans*, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 651–656.
9. Pusheva, M.A., Pitryuk, A.V., and Berestovskaya, Yu.Yu., Metabolic Peculiarities of the Extremely Alkaliphilic Sulfate-Reducing Bacteria *Desulfonatronum lacustre* and *Desulfonatronovibrio hydrogenovorans*, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 657–663.
10. Ugarova, N.N., Brovko, L.Yu., Trdatyan, I.Yu., and Rainina, E.I., Bioluminescent Methods of Analysis in Microbiology, *Prikl. Biokhim. Mikrobiol.*, 1987, vol. 23, no. 1, pp. 14–24.
11. Stanley, P.E. and Williams, S.G., Use of the Liquid Scintillation Spectrometer for Determining Adenosine Triphosphate by the Luciferase Enzyme, *Anal. Biochem.*, 1969, vol. 29, pp. 381–392.
12. Ljungdahl, L.G., The Acetyl-CoA Pathway and the Chemiosmotic Generation of ATP during Acetogenesis, *Acetogenesis*, Drake, H.L., Ed., New York: Chapman & Hall, 1994, pp. 63–87.
13. Pusheva, M.A., Pitryuk, A.V., and Netrusov, A.I., Inhibitory Analysis of the Energy Metabolism of the Extremely Haloalkaliphilic Homoacetic Bacterium *Natroniella acetigena*, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 647–650.
14. Odom, J.M. and Peck, H.D., Hydrogenases, Electron-Transfer Proteins, and Energy Coupling in the Sulfate-Reducing Bacteria *Desulfovibrio*, *Annu. Rev. Microbiol.*, 1984, vol. 38, pp. 551–592.
15. Kreke, B. and Cypionka, H., Role of Sodium Ions for Sulfate Transport and Energy Metabolism in *Desulfovibrio salexigenes*, *Arch. Microbiol.*, 1994, vol. 161, pp. 55–61.
16. Zavarzin, G.A., Zhilina, T.N., and Kevbrin, V.V., The Alkaliphilic Microbial Community and Its Functional Diversity, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 579–599.