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EXPERIMENTAL ARTICLES

Comparative Study of the Energy Metabolism of Anaerobic Alkaliphiles from Soda Lakes

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Abstract—We investigated the influence of inhibitors of energy metabolism and ionophores on the growth and formation of metabolic products in alkaliphilic anaerobes characterized by various catabolism types. It was shown that blockage of oxidative phosphorylation by the addition of *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of F_1F_0 ATP synthase, resulted in a complete arrest of the growth of the acetogenic bacterium *Tindallia magadiensis* with arginine as an electron acceptor. In the presence of pyruvate, substrate-level phosphorylation occurred. The methylotrophic methanogenic archaebacterium *Methanosalsus zhilinae* did not grow with DCCD and vanadate, an inhibitor of E_1E_2 ATPase, suggesting the presence of two ATPase types in this species. In the saccharolytic alkaliphiles *Halonatronum saccharophilum, Amphibacillus tropicus*, and *Spirochaeta alkalica* (which are characterized by different pH optima), the contribution of the H^+ gradient to the energy metabolism and, presumably, to the maintenance of the intracellular pH level decreased with an increase in the degree of alkaliphily. Based on the data of an inhibitor assay using protonophores, monensin, and amiloride, we suggest that all of the bacteria tested depend on H^+ and Na^+ gradients. The Na^+/H^+ antiport appears to be a universal mechanism of regulating the intracellular pH level and the interaction between the Na^+ and H^+ cycles in bacterial cells cultivated under alkaline conditions.

Key words: alkaliphiles, anaerobes, homoacetogens, methanogens, fermenters, bioenergetics, ATPases.

Studies on extreme haloalkaliphilic anaerobic prokaryotes are of considerable interest in terms of the bioenergetics of the growth of microorganisms at pH values over 9 [1]. The main challenge faced by extreme alkaliphilic bacteria is the necessity to maintain the intracellular pH level and the bioenergetic status of their cells in a medium with a low proton concentration. Membranes of alkaliphiles are characterized by an "inside-out" proton gradient. Accordingly, a proton transferred from a cell during, e.g., respiration, can do no useful work upon reentering the cell because it has to move against the proton gradient. A possible scenario of adapting to growth under alkaline conditions implicates the employment of a sodium-dependent energy transduction cycle. The generation and utilization of Na^+ gradients in the membranes of marine alkalitolerant, moderately halophilic vibrios was described [2]. A Na^+ cycle was also revealed in some neutrophilic anaerobes, including aceto- and methanogenic species [3].

An inhibitor assay of the growth and biosynthetic activities of neutrophilic, halophilic, and alkaliphilic prokaryotes allows us to estimate the contributions of oxidative and substrate-level phosphorylation and to elucidate the roles of H^+ and Na^+ gradients in bacteria characterized by various metabolism types.

It was shown by us earlier that the energy metabolism of extreme halophiles, such as the acetogenic bacteria *Natroniella acetigena* and *Natronincola histidinovorans* and sulfate-reducing bacteria from soda lakes, depends on both the proton and the sodium electrochemical gradients and involves electron transfer–level phosphorylation that is coupled with the electron transport via membrane ATP synthase–dependent chemiosmosis, regardless of the substrate employed [4–6].

We also isolated the acetogenic ammonifier *Tindallia magadiensis* (formerly termed *T. magadii*) [7], the methylotrophic methanogen *Methanosalsus zhilinae* [8], and saccharolytic anaerobic fermenters [9, 10].

T. magadiensis is an alkaliphilic, strictly anaerobic, non-spore-forming bacterium, whose energy metabolism is based on the utilization of a number of amino acids and other organic acids. The growth of the bacterium depends on Na^+; the optimum is attained at a $NaHCO_3$ concentration of 4–8%. In contrast to haloalkaliphilic bacteria, this bacterium does not require NaCl for growth. This moderately alkaliphilic microorganism (with a pH optimum of 8.5) is of considerable interest in terms of sodium energetics. The growth of the methylotrophic methanogen *M. zhilinae*, which grows on methanol with a pH optimum at pH 9.2, is obligately dependent on the addition of Na^+ and HCO_3^-.

Prokaryotic saccharolytic fermenters chiefly carry out fermentation processes involving substrate degradation products as reducing equivalent acceptors. The

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energy metabolism of such organisms is based on substrate-level phosphorylation. Prerequisite for the transfer of substances, flagellum rotation, and the maintenance of the intracellular pH level are ion gradients that form in fermenter cells at the expense of ATP hydrolysis involving ion-transporting ATPases [2]. The generation of the ion gradient at the expense of ATP hydrolysis can be performed not only by fermenters; however, only fermenters lack the ATP synthase function of this enzyme, probably due to the presence of another energy-supplying mechanism based on substrate-level phosphorylation [2, 11].

The goal of this study was to investigate the influence of protonophores and inhibitors on the growth of representatives of alkaliphilic anaerobic prokaryotes characterized by various metabolism types and on the formation of the main metabolic products in their cells.

MATERIALS AND METHODS

This study used pure cultures of the anaerobic alkaliphiles *Tindallia magadiensis* Z-7934^T, *Methanosalsus zhilinae* Z-7936^T, *Spirochaeta alkalica* Z-7491^T, *Halonatronum saccharophilum* Z-7986^T, and *Amphibacillus tropicus* Z-7792^T that were isolated from Lake Magadi (Kenya) and donated by T.N. Zhilina and V.V. Kevbrin.

T. magadiensis was cultivated under strictly anaerobic conditions at 37°C, pH 8.5, on a mineral medium of the following composition (g/l): $NaHCO_3$ 40, KH_2PO_4 0.3, NH_4Cl 0.5, $Na_2S \cdot 9H_2O$ 0.5, trace element solution (1 ml), 0.04% resazurin (2 ml), and yeast extract 0.2 g/l. Arginine (2 g/l) or pyruvate (5 g/l) were used as substrates. To remove oxygen from the medium, it was boiled and thereupon purged with argon for 15 min. Upon cooling, the medium was supplemented with $NaHCO_3$, yeast extract, resazurin (an Eh indicator) and a reductant (Na_2S). The medium was thereupon transferred under argon to Hungate tubes or flasks. It was sterilized in an autoclave for 30 min at 120°C. A culture harvested during the midlogarithmic growth phase served as the inoculum (3%, v/v) [7].

M. zhilinae was grown (pH 9.2) on a medium with methanol (0.5%) of the following composition (g/l): K_2HPO_4 0.2, KCl 0.2, NH_4Cl 1.0, $NaHCO_3$ 32.0, trace element solution 1 ml, vitamin solution 2 ml, 0.04% resazurin 2 ml, and $Na_2S \cdot 9H_2O$ 0.5 [7]. *S. alkalica* was grown (pH 9.0) anaerobically on a medium containing (g/l) Na_2CO_3 2.0, $NaHCO_3$ 4.5, NaCl 48.0, NH_4Cl 0.5, KH_2PO_4 0.2, yeast extract 0.5, sucrose 5.0, trace element solution 1 ml, vitamin solution 1 ml, and 0.04% resazurin 2 ml [8]. The following medium was used for cultivating *A. tropicus* and *H. saccharophilum* (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, sucrose 5.0, trace element solution 1 ml, vitamin solution 1 ml, and 0.04% resazurin 2 ml (pH 9.7) [10].

The composition of the trace element solution was as follows (mg/l): $MnCl_2 \cdot 4H_2O$ 720, $(NH_4)_2SO_4 \cdot 6H_2O$ 400, $FeSO_4 \cdot 7H_2O$ 200, $CoCl_2 \cdot 6H_2O$ 200, $ZnSO_4 \cdot 7H_2O$ 200, $NiCl_2 \cdot 6H_2O$ 100, $CuSO_4 \cdot 5H_2O$ 20, $AlK(SO_4)_2 \cdot 12H_2O$ 20, H_3BO_4 20, $Na_2MoO_4 \cdot 4H_2O$ 20, and EDTA 1 g/l. The composition of the vitamin solution was as follows (mg/l): 4-aminobenzoic acid 25, D-biotin 100, nicotinic acid 25, calcium pantothenate 25, pyridoxine hydrochloride 25, folic acid 10, riboflavin 25, cyanocobalamin 0.5, and lipoic acid 25.

The growth of the bacteria was determined by (i) measuring optical density at 600 nm by means of a Specol-11 spectrophotometer (Germany) directly in tubes or in 1-cm cuvettes; (ii) counting the cells directly; and (iii) measuring the intracellular protein content after precipitating the cells by centrifugation at 14500 *g* for 3 min with subsequent hydrolysis in 1 N NaOH (the protein was determined by the method of Lowry *et al.* [12]).

Cell suspensions of *S. alkalica* were obtained by centrifuging actively growing cells at 4550 *g* for 60 min. The cells were thereupon washed and resuspended in a mineral cultivation medium that contained 3 mM dithiothreitol, 2 mM $MgCl_2$, and 0.5% ethanol. The final cell protein content of the suspensions was 0.6–1 mg/ml. The cell suspensions were prepared under anaerobic conditions.

Acetate was determined with a Chrom-5 gas chromatograph (Czech Republic) with a flame-ionization detector. Separation was performed at 160°C on a 0.9 m × 3 mm glass column filled with Chromosorb-101 (Sigma, USA). Argon was the carrier gas. Acetate was determined by acidifying culture liquid with concentrated HCl to pH 2; thereupon, 2 µl of the sample were applied onto the column. Hydrogen was determined with an LKhM-80 chromatograph equipped with a katharometer. Argon was the carrier gas. Separation was performed on a 0.75 m × 3 mm column filled with 5A molecular sieve.

Hydrogenase activity was determined by monitoring benzyl viologen reduction under H_2 at 600 nm [4].

The metabolic inhibitors *N,N'*-dicyclohexylcarbodiimide (DCCD), monensin, 3,5-di-tert-butyl-4-hydroxybenzidylidenyl malonitrile (SF-6847), amiloride, *n*-trifluorometoxycarbonylcyanide phenylhydrazone (FCCP) (Serva, Germany), and rhodamine 6G (Reakhim, Russia) were added in the form of ethanol solutions to the cultivation medium. Equivalent ethanol amounts were added to the control cultures. Vanadate was added in the form of an aqueous solution.

RESULTS AND DISCUSSION

T. magadiensis. Fig. 1 contains our data on the influence of inhibitors and ionophores (that were introduced immediately after inoculation) on biomass yield and acetogenesis in *T. magadiensis* after 3 days of growth on arginine (during the early stationary phase).

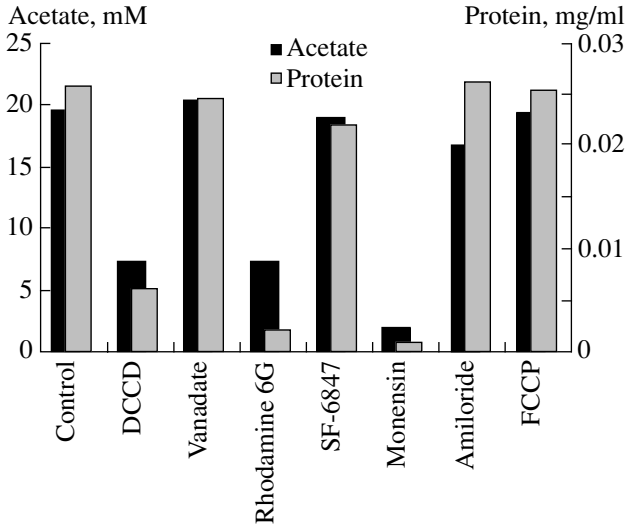

Fig. 1. Effect of inhibitors and ionophores on biomass yield and acetogenesis in *T. magadiensis* grown on medium with arginine (2 g/l) after 3 days of cultivation (the beginning of the stationary phase). Additions: 500 μM DCCD, 50 μM vanadate, 3 μM rhodamine 6G, 3.2 μM SF-6847, 10 μM monensin, 50 μM amiloride, and 50 μM FCCP.

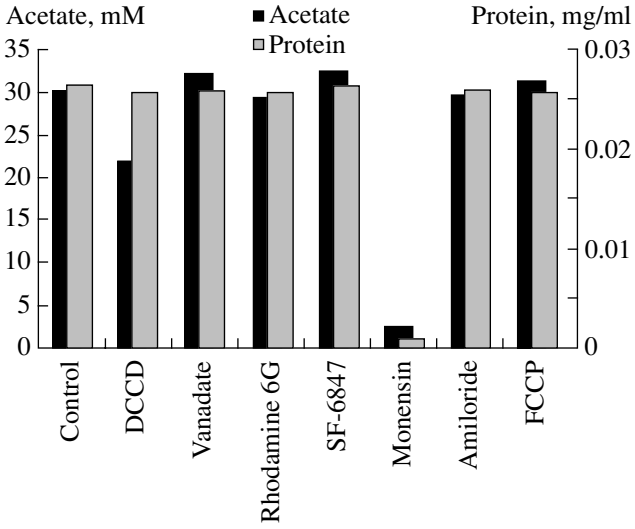

Fig. 2. Effect of inhibitors and ionophores on biomass yield and acetogenesis in *T. magadiensis* grown on medium with pyruvate (5 g/l) after 3 days of cultivation (the beginning of the stationary phase). See legend to Fig. 1 for the concentrations of the inhibitors and ionophores.

T. magadiensis growth and acetogenesis were suppressed by 500 μM DCCD, an inhibitor of ΔpH- or ΔpNa-coupled ATP hydrolysis and synthesis. Using arginine as the substrate implies its involvement in the ornithine cycle and conversion to carbamonyl phosphate with subsequent CO_2 and NH_3 formation. The latter reaction implicates ATP formation from ADP and P_i involving ATP synthase [13]. CO_2 is subsequently incorporated into the acetyl-CoA pathway that results in the formation of acetate as the final product. The ATPase of *T. magadiensis* belongs to the F_1F_0 type because vanadate, an E_1E_2 inhibitor, failed to suppress arginine-dependent growth and acetogenesis at a concentration of 50 μM.

The ion specificity of the ATPase of *T. magadiensis* was assessed using rhodamine 6G, which effectively suppresses ΔpH-dependent ATP synthesis in mitochondria and *Methanobacterium thermoautotrophicum* without affecting ΔpNa-dependent ATP synthesis. The site of action of rhodamine 6G is the membrane-embedded part of the ATPase complex of *M. thermoautotrophicum* [14]. The inhibitory effect of 3 μM rhodamine 6G on *T. magadiensis* growth and acetogenesis compares with that of 500 μM DCCD. This seems to support the idea that arginine oxidation during acetogenesis is coupled with ATP generation via a chemiosmotic mechanism that involves a ΔpH-dependent ATP synthase.

In contrast, DCCD did not affect *T. magadiensis* growth with pyruvate as the substrate, although it inhibited acetate synthesis by almost 30% (Fig. 2). Hence, growth was decoupled from acetogenesis in the presence of DCCD. Rhodamine 6G did not affect growth and acetogenesis. Pyruvate in acetogenic bacteria is oxidized by pyruvate–ferredoxin oxidoreductase to acetyl-CoA, reduced ferredoxin, and CO_2, which is incorporated into the Wood–Ljungdahl pathway. Acetyl-CoA is converted to acetate in an acetyl phosphate–involving process [3]. In this case, the Wood–Ljungdahl pathway serves as an electron sink, enabling the bacteria to synthesize ATP at the expense of substrate-level phosphorylation. Presumably, oxidative phosphorylation is involved in energy generation during acetogenesis, because DCCD partially inhibited acetogenesis in pyruvate-grown *T. magadiensis*.

Biomass synthesis in acetogenic bacteria is tightly linked to acetogenesis, and the acetate–biomass unit ratio directly reflects the status of the cell energetics. It was shown that 700 μM DCCD suppressed the growth and acetogenesis of the neutrophilic acetogenic bacterium *Thermoanaerobacter kivui* only if it grew on H_2 autotrophically [15]. Similar data were obtained by us with the halophilic acetogen *Acetohalobium arabaticum*, which grew autotrophically on media with H_2 or CO. A complete suppression of growth and acetogenesis was achieved upon increasing the DCCD concentration from 700 to 1400 μM. The growth of this bacterium was insensitive to this inhibitor on media with lactate or formate [16]. However, 500 μM DCCD and protonophores completely inhibited the growth and acetogenesis of the acetogenic bacterium *N. acetigena* with lactate or ethanol, suggesting an involvement of the proton cycle and ATPase in H^+ translocation into the cell for the purpose of maintaining pH homeostasis under alkaline conditions [5, 6]. It was shown by us earlier that the growth and sulfidogenesis of the alka-

Table 1. Effect of inhibitors on the growth of *M. zhilinae* on a methanol-containing medium after 7 days of cultivation (beginning of the stationary phase)

Inhibitor, μM	Growth inhibition, %
Control	0
DCCD, 500	100
Vanadate, 50	100
SF-6847, 3	60
Monensin, 50	100
Amiloride, 50	64
Rhodamine 6G, 3	100

liphilic sulfate-reducing bacterium *Desulfonatronum lacustre* were totally suppressed by 500 μM DCCD and only partly inhibited by rhodamine 6G. Accordingly, the bacterium can use the primary Na^+ gradient for synthesizing ATP by means of the Na^+ ATPase, while H^+ ATPase is inhibited by rhodamine 6G [4]. These results were corroborated by the discovery of Na^+-dependent ATP synthesis (suppressed by 50 μM monensin) in *D. lacustre* [6]. Monensin is known to be an electroneutral electrogenic Na^+/H^+ antiporter, whose addition to cells or vesicles affects the membrane potential and the intracellular Na^+ concentration [17].

The growth and acetogenesis of *T. magadiensis* with arginine and pyruvate are sensitive to 10 μM monensin, which collapse the Na^+ and H^+ gradients across the membrane. Similar data were obtained by us for the haloalkaliphilic bacterium *N. acetigena*, whose growth and acetogenesis were completely inhibited by monensin (14 μM) on all tested substrates [5].

The protonophore SF-6847 did not affect the growth and acetogenesis of *T. magadiensis* on pyruvate and caused a 15% growth inhibition with arginine. It had virtually no effect on acetate synthesis. The growth and acetogenesis of *T. magadiensis* on the tested substrates were resistant to the protonophore FCCP and to amiloride.

M. zhilinae. Table 1 contains our data on the influence of inhibitors on the growth of *M. zhilinae* on the medium with methanol. DCCD, an F_1F_0 ATPase inhibitor, and vanadate, an E_1E_2 type ATPase inhibitor, totally suppressed the growth and methanogenesis of *M. zhilinae*. The conclusion can be drawn that methane oxidation is coupled in this methanogen with ATP synthesis via membrane phosphorylation that involves both the F_1F_0 and E_1E_2 ATPases. It was shown that the E_1E_2 ATPase of *Methanosarcina mazei* can perform ATP hydrolysis that is coupled with ion extrusion from the cell [18]. Unfortunately, an inhibitor assay of growth dynamics does not clarify whether a V_0V_1 type ATPase operates in the cells involved. According to data on the purified Na^+-translocating V ATPase of *Enterococcus hirae*, this enzyme was not inactivated by DCCD in the presence of Na^+ [19]. The Na^+ content in the medium for *M. zhilinae* is over 380 mM. In addition, amiloride, an inhibitor of a large number of Na^+-dependent transport systems that involve the operation of Na^+ channels, a Na^+/H^+ antiport, and flagellum rotation, has no effect on V type ATPases, while the growth of *M. zhilinae* is suppressed by 64% by amiloride. The inhibition of ΔpH-dependent ATP synthesis by rhodamine 6G resulted in a total suppression of the growth of *M. zhilinae.* The protonophore SF-6847 inhibited the growth of *M. zhilinae* by 60%, whereas collapsing the Na^+ gradient with monensin caused a complete arrest of growth.

The data of the inhibitor assay performed with *M. zhilinae* suggest that the energy metabolism of this organism combines the H^+ and the Na^+ cycles. The growth of the tested archaebacterium on methanol involves membrane phosphorylation. Two different types of ATPases operate in the cells, and at least one of them translocates H^+.

Methanogenesis in nonhalophilic methanogens is tightly coupled with the generation of both $\Delta\mu Na^+$ (resulting from the methyltetrahydromethanopterine-CoM-SH-methyl transferase reaction) and $\Delta\mu H^+$ (resulting from the CoM-mercaptoheptanoylthreonine-phosphate–heterodisulfide reductase reaction) [3]. In the marine methanogen *M. voltae*, both pumps (methyl transferase and heterodisulfide reductase) employ Na^+ [20]. Na^+ ATPase was also revealed in the halophilic methanogen *Methanohalophilus halophilus* and the thermophile *M. thermoautotrophicum* [14].

M. mazei Gö 1 uses these gradients for ATP synthesis that involves F_1F_0 and A_1A_0 ATPases [18]. However, the Na^+ gradient does not fuel ATP synthesis in *Ms. barkeri* cells, but it results in the generation of an H^+ gradient via a Na^+/H^+ antiporter. The H^+ ATPase is the only enzyme that catalyzes membrane ATP synthesis in *Ms. barkeri* [20]. Another strategy of using ion gradi-

Table 2. Effect of ionophores and inhibitors of energy metabolism on the growth of *A. tropicus* and *H. saccharophilum* after 1 day of growth (beginning of the stationary phase)

Species	Growth conditions	Growth inhibition, %				
		DCCD, 500 μM	SF-6847, 3 μM	Monensin, 15 μM	Amiloride, 50 μM	Rhodamine 6G, 3 μM
A. tropicus	pH 9.5, 7% NaCl, glucose	100	4	100	25	93
H. saccharophilum	pH 8.5, 10% NaCl, glucose	100	100	100	32	72

ents occurs in the marine species *M. voltae*, which employs two Na^+ pumps as catalysts of exergonic reactions during methanogenesis. Hence, ATP synthesis is also Na^+-dependent in this methanogen [21].

Thus, we conclude that methanogens use various membrane mechanisms to generate ion gradients and to employ their energy, depending on the thermodynamic feasibility of these bioenergetic mechanisms under various environmental conditions.

Saccharolytic fermentation-capable bacteria. Figure 3 demonstrates the influence of inhibitors and ionophores on the formation of the fermentation products (hydrogen and acetate) by cell suspensions of the alkaliphile *S. alkalica* (pH 9.0). H_2 evolution by *S. alkalica* cells involved a hydrogenase whose activity was 0.18 μmol/(min mg protein). DCCD partially and totally inhibited the formation of H_2 and acetate at concentrations of 250 and 500 μM, respectively. Rhodamine did not affect the rate of formation of these products, and, therefore, H^+ ATPase is unlikely to be involved in the energy metabolism of *S. alkalica*. Collapsing the Na^+ and H^+ gradients by monensin resulted in a complete suppression of the generation of the products. The Na^+/H^+ antiport inhibitor amiloride failed to exert any significant influence on these processes. The protonophore SF-6847 inhibited H_2 and acetate formation by approximately 50%. The results obtained suggest that the metabolism of *S. alkalica* depends on both the Na^+ and the H^+ gradients. However, these results do not allow us to unambiguously clarify the issue of the mechanisms of pH homeostasis in *S. alkalica* cells at pH 9.0.

Of special interest was a comparative study of the effects of inhibitors and ionophores on the growth of the fermenting bacteria *A. tropicus* and *H. saccharophilum*, which grow with 7% NaCl (pH 9.5) and 10% NaCl (pH 8.5), respectively. DCCD completely inhibited the growth of the tested organisms (Table 1). Rhodamine 6G suppressed the growth of *A. tropicus* and *H. saccharophilum* by 93 and 72%, respectively, suggesting the involvement of an F_1F_0-type ATPase in the maintenance of intracellular pH homeostasis. Presumably, this enzyme plays a more important role in *A. tropicus* than in *H. saccharophilum*.

Monensin totally suppressed the growth of both saccharolytic bacteria. The protonophore SF-6874 produced different effects in these two organisms. It completely inhibited the growth of *H. saccharophilum* and only insignificantly decelerated the growth of *A. tropicus*. Amiloride exerted the same influence on the growth of both saccharolytic species. Their growth was suppressed by approximately 30% compared to the control cultures (Table 2).

Hence, we conclude that the proton gradient plays a limited role in the metabolism of the more alkaliphilic saccharolytic species *A. tropicus.* In this organism, the Na^+ cycle performs a key function in the maintenance of the intracellular pH level and the transfer of substances into the cell. However, the data of an inhibitor assay of the growth of *H. saccharophilum* (pH 8.5) indicate that the metabolism of this bacterium depends both on the Na^+ gradient and on the H^+ gradient. In both fermenters, the F_1F_0-type H^+ ATPase carries out ATP hydrolysis that is coupled with outward H^+ transport.

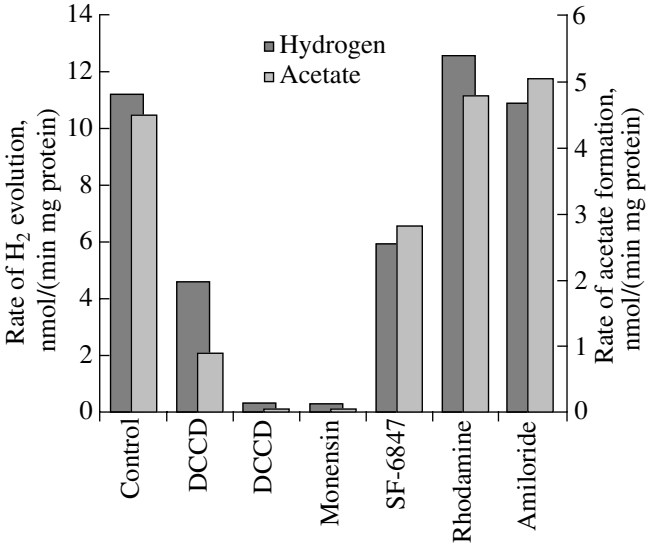

Fig. 3. Effect of inhibitors and ionophores on acetate production and hydrogen evolution by cell suspensions of *Spirochaeta alkalica* grown on medium with sucrose (5 g/l) after 1 day of cultivation (the beginning of the stationary phase). Additions: 250 μM DCCD, 500 μM DCCD, 50 μM monensin, 3 μM SF-6847, 3 μM rhodamine 6G, and 50 μM amiloride.

The results of the inhibitor assay of anaerobes from the alkaliphilic prokaryotic community, performed with growing cultures and cell suspensions, imply that their energetics is based on the operation of a Na^+ pump and/or a H^+ pump. The Na^+/H^+ antiport is essential for maintaining a low intracellular pH level compared to the ambient pH level if the bacteria grow on alkaline media. This fact was established with extremely alkaliphilic bacilli [22].

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