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REVIEW

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# Energy Metabolism in Halophilic and Alkaliphilic Acetogenic Bacteria

E. N. Detkova<sup>1</sup> and M. A. Pusheva

*Winogradsky Institute of Microbiology, Russian Academy of Sciences,  
pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

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**Abstract**—This review summarizes the recent data on the energy metabolism of acetogenic bacteria isolated from saline and soda lakes. It provides a general description of saline and soda lakes as microbial habitats, performs a comparative analysis of the energy metabolism of neutrophilic acetogens, considers the adaptation strategies of halophilic and alkaliphilic acetogens to high salinity and high pH values, while also discussing the mechanisms of energy accumulation under auto- and organotrophic conditions.

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Extremophilic microorganisms, especially extreme halophiles and haloalkaliphiles, are currently receiving increased attention in the literature [1–10]. This is due to the theoretical importance of disclosing the biochemical mechanisms that enable organisms to grow at high salinities and pH values over 9 and to the biotechnological potential of haloalkaliphiles that produce alkaline enzymes and polymers and can be used for wastewater treatment. Natural hypersaline and alkaline habitats exist in all continents, but they prevail in regions characterized by a warm and dry climate. Saline and soda lakes are impressive examples of relevant natural habitats. The salinity values of such lakes range from 5% to the saturation level (30% and above). Na<sup>+</sup> and Cl<sup>−</sup> are predominant ions in saline lakes, while soda lakes also contain carbonate/bicarbonate ions [1, 11–13]. These niches are inhabited by microbial communities that are adapted to such ecological conditions. The issue concerns the strategies of adaptation of microorganisms to extreme environmental conditions, because high salt concentrations and pH values exert, as a rule, a deleterious influence on their proteins and other macromolecules. Because halo- and alkaliphiles cope with a number of specific stress factors, their energy metabolism should differ from that of neutrophiles. Attaining the complex goal of maintaining the intracellular pH level and resisting osmotic stress requires systemic adaptation to extreme conditions [4]. This adaptation is based on the properties of cell lipids, proteins, and other macromolecules, which involves bioenergetic processes and the regulation of intracellular ion

composition. This review presents our own experimental data in conjunction with the information available in the literature concerning bioenergetics and osmoadaptation of halophilic and haloalkaliphilic microorganisms. It focuses on the studies with representatives of anaerobic acetogenic bacteria from extreme habitats. A large number of recent studies deal with neutrophilic acetogens and their energy metabolism. However, the adaptational mechanisms and energy metabolism of acetogens from extreme niches have not yet been understood, because such habitats have not been sufficiently investigated until recently in terms of acetogenesis processes and relevant microorganisms.

## SALINE AND SODA LAKES AS HABITATS

**General characterization of saline lakes.** Saline ecosystems are common in nature. The water in oceans and seas contains about 35 g/l of dissolved salts. Higher salt concentrations often occur in coastal areas including saline marshes and lagoons if evaporation of water is a more rapid process than its dilution by bulk seawater. Still higher salt concentrations (up to the NaCl saturation level) were found in natural intracontinental saline lakes such as the Dead Sea (Israel/Jordan) and the Great Salt Lake (Utah, USA) [1]. The properties of hypersaline bodies of water as habitats for halophilic and halotolerant prokaryotes are conditional on the total salt concentration. However, ion composition also plays a key role with respect to the biotope function of the body of water involved. The ion composition of the brine resulting from seawater evaporation (thalassophilic saline) resembles that of seawater per se, at least

<sup>1</sup> Corresponding author; e-mail: detkovkate@rambler.ru

during the initial evaporation stage.  $\text{Na}^+$  and  $\text{Cl}^-$  are the predominant ions. Prolonged evaporation results in ion composition changes caused by the precipitation of gypsum ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) and other minerals (total salt concentrations exceeding 100–120 g/l). Subsequent sedimentation of  $\text{NaCl}$  in the form of halite entails additional changes: the relative potassium and magnesium concentrations increase. Thalassophilic brine is characterized by neutral or slightly alkaline pH values (7–8). In contrast, there are hypersaline niches whose ion composition significantly differs from that of seawater (athalassophilic saline), exemplified by the Dead Sea. It is dominated by divalent cations (the  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  concentrations are 1.89 and 0.45 M, respectively). The  $\text{Na}^+$  and  $\text{K}^+$  concentrations are 1.56 and 0.2 M, respectively [8]. Monovalent  $\text{Cl}^-$  and  $\text{Br}^-$  anions account for over 99.9% of the total amount of all anions. The water pH value of the Dead Sea is relatively low (~5.8–6.0).

**Soda lakes and their origins.** Soda lakes are widespread bodies of water containing athalassophilic alkaline saline. The formation of soda lakes and athalassophilic saline lakes is generally governed by the same laws, with the main difference being that soda lakes contain bicarbonate as the dominant anion. Alkalization is facilitated by geological and climatic factors, particularly by a lacking amount of significant  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the environment. The dominant continental geochemical process, carbon dioxide-dependent leaching of rocks by meteoric waters, in combination with intense evaporation, forms undrained soda lakes [14]. Wind erosion and biological processes result in the accumulation of carbon dioxide-saturated water on and near the surface of the lake. Thus, carbonate-bicarbonate solution forms, which leaches minerals from the adjacent rocks. Ion accumulation caused by evaporation shifts the  $\text{CO}_2/\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  equilibrium. The water is rapidly saturated by  $\text{Ca}^{2+}$  in most habitats, resulting in the precipitation of calcite ( $\text{CaCO}_3$ ) that is often accompanied by magnesite ( $\text{MgCO}_3$ ) and dolomite ( $\text{MgCa}(\text{CO}_3)_2$ ). Hence, carbonate is removed from the solution if the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentration exceeds the  $\text{CO}_3^{2-}$  concentration, and a neutral lake forms instead of an alkaline lake. If, conversely, the  $\text{CO}_3^{2-}$  concentration exceeds the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentration the lake becomes alkaline, and  $\text{Na}^+$  typically is the dominant cation [12, 15, 16]. The resulting habitats are both alkaline and saline. Monovalent cations are the prevalent ions in the water. Due to the high pH values (10–11 and above), the solubility of divalent cations, such as  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ , is very low, and their concentrations may be too insignificant to be detected. Apart from carbonate and bicarbonate ions, chloride and sulfate also account for a significant share of the total anion concentration in lakes of this type [16].

## MECHANISMS OF ADAPTATION OF MICROORGANISMS TO HIGH SALT CONCENTRATIONS AND HIGH pH VALUES

The life of microbes at high salt concentrations is less “profitable” in terms of energetics than at neutral pH values [5]. Most halophilic and halotolerant microorganisms meet the osmolarity challenge by maintaining high intracellular concentrations of organic osmoregulators, which incurs considerable energy expenses (the “compatible osmoregulators” strategy). As we know the biochemical pathways of the biosynthesis of these compounds, we can estimate the energy expenses, proceeding from the number of ATP molecules required for the synthesis of one osmoregulator molecule. However, two specialized groups of microorganisms—the aerobic halophilic archaeobacteria of the order *Halobacteriales* and the anaerobic halophiles of the order *Halanaerobiales*—do not synthesize osmoregulators. They accumulate intracellular  $\text{Na}^+$  (up to 1 M) and  $\text{K}^+$  (up to 4–5 M) to maintain the intracellular turgor, and their proteins are adapted to such high salt concentrations (“salt inside” strategy). The proton electrochemical gradient ( $\Delta\mu_{\text{H}^+}$ ) across the cytoplasmic membrane is the primary energy source for  $\text{Na}^+$  extrusion and  $\text{K}^+$  accumulation inside the cells of most halophiles. The gradient forms at the expense of either the electron transfer in the respiratory chain (during aerobic growth of representatives of the order *Halobacteriales*) or ATP that is synthesized by substrate phosphorylation and converted to  $\Delta\mu_{\text{H}^+}$  by means of a membrane-bound ATPase (the F type  $\text{H}^+$ -ATPase in bacteria and the A type  $\text{H}^+$ -ATPase in halophilic archaeobacteria). In addition,  $\Delta\mu_{\text{H}^+}$  can be generated using light energy in a bacteriorhodopsin-involving process (in bacteriorhodopsin-containing halobacteria) [5, 8, 17]. The sodium gradient ( $\Delta\mu_{\text{Na}^+}$ ) forms at the expense of the proton gradient by means of electroneutral or electrogenic  $\text{Na}^+/\text{H}^+$  antiporters. It can function as the driving force of some endergonic processes in the cell.

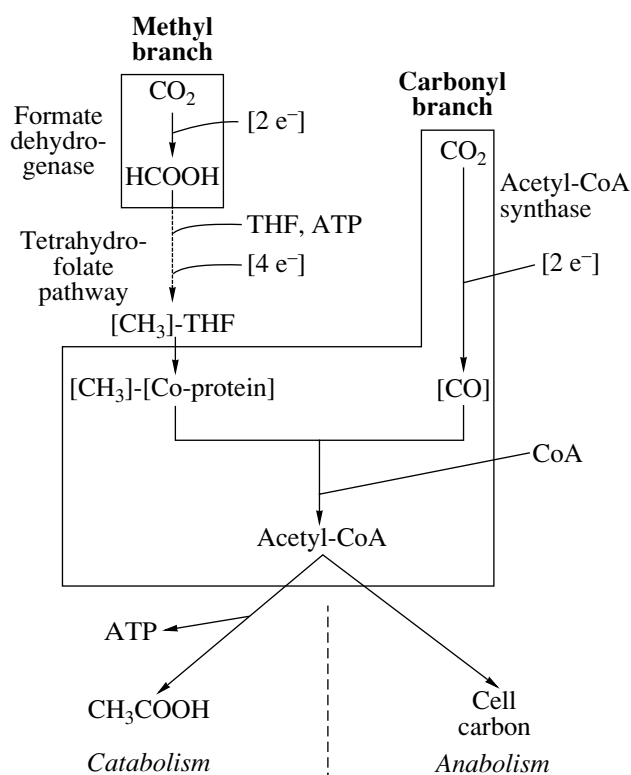
In contrast to hypersaline lake inhabitants, microorganisms in a soda lake are exposed to double stress caused by the high salt concentrations and high ambient pH values. Apart from the problem of osmoadaptation, alkaliphiles develop in media with high pH values and, accordingly, low proton concentrations have to cope with maintaining pH homeostasis inside the cell [18]. Energy metabolism is based on inward ion transfer with concomitant ATP synthesis. The proton-motive force generated by proton pumping and respiration provides for ATP synthesis both in neutro- and alkaliphiles. However, the cytoplasmic pH values are maintained on a near-neutral level in alkaliphile cells because of the operation of a  $\text{Na}^+/\text{H}^+$  antiporter. Accordingly, a straightforward chemiosmotic mechanism cannot account for the generation of a proton-motive force

across the cytoplasmic membrane in alkaliphilic bacteria, which grow at high pH values. Krulwich *et al.* suggested a “localized gradient” hypothesis invoking the partial localization of both the ATP synthase and the respiratory chain components in the membrane [19]. Research on the bioenergetics of alkaliphiles resulted in the discovery of a sodium cycle that can effectively replace the proton cycle [20, 21]. The low proton concentration in the medium causes kinetic problems for all proton-involving systems. A more serious problem arises if the external  $[H^+]$  concentration is lower than the internal  $[H^+]$  concentration. In this system, the electrical potential  $\Delta\psi$  (with the negative charge inside the cell) is counterbalanced by the  $\Delta pH$  with an opposite direction. The protons extruding from the cell, as a result of, e. g., respiration, cannot do useful work when re-entering the cytoplasm, because they should move against the concentration gradient. Replacing protons by  $Na^+$  ions was a radical solution to this problem. Secondary  $Na^+/H^+$  antiport is the central mechanism involved in  $Na^+$  extrusion. The chemiosmotic reverse  $\Delta pH$  gradient is built up at the expense of the electrochemical  $Na^+$  gradient [7, 22]. The antiporter substitutes protons for sodium ions in a transmembrane electrogenic process, which results in extruding  $Na^+$  ions at the expense of the electrochemical proton potential.

## NEUTROPHILIC ACETOGENS

**The Wood–Ljungdahl acetyl-CoA pathway and energy conservation.** Acetogenic bacteria represent a group of obligate anaerobes that use the Wood–Ljungdahl acetyl-CoA pathway for the reductive synthesis of acetate from  $CO_2$  for the purpose of energy accumulation and the synthesis of cell carbon compounds [23]. These bacteria are ubiquitous in anoxic habitats, playing a major role in the global carbon cycle. Homoacetogens are the acetogens that form acetate as the sole reduced terminal product from relevant substrates. Following are the main metabolic properties of these organisms: (i) utilization of inorganic substrates ( $H_2/CO_2$ ) or  $CO/CO_2$  as the sole carbon and energy sources under strictly anaerobic conditions; (ii) capacity for stoichiometric conversion of some sugars to acetate; (iii) utilization of aromatic compounds under acetogenic conditions [23]. Most, but not all, acetogens possess these three properties.

The acetyl-CoA pathway can be subdivided into the methyl and carbonyl stage (Fig. 1) [24, 25]. The methyl stage implicates  $CO_2$  reduction to formate, which is subsequently converted to the methyl group of methyltetrahydrofolate (methyl-THF). The second stage involves the formation of a one-carbon intermediate from  $CO$ ,  $CO_2$ , and  $H_2$ . It is thereupon combined with CoA and methylated corrinoid protein to form acetyl-CoA. The stoichiometry of the  $H_2$ -dependent acetogenesis is as follows:



**Fig. 1.** The autotrophic acetyl-CoA pathway [25].  $e^-$ , reducing equivalent; THF, tetrahydrofolate; [Co-protein], corrinoid protein; CoA, coenzyme A.

The standard change in free energy is about  $-100$  kJ/mol acetate for this reaction.

Under autotrophic conditions, with acetogens growing in the  $H_2 + CO_2$  mixture as the sole carbon and energy source, the Wood–Ljungdahl pathway supplies metabolic energy [23, 26, 27]. One ATP molecule is consumed to activate formate (Table 1, reaction 2) and one ATP molecule is generated via substrate phosphorylation in the acetate kinase reaction (Table 1, reaction 10) [28], therefore a net ATP accumulation does not occur at the substrate phosphorylation level. In this system,  $CO_2$  reduction to acetate is coupled to oxidative phosphorylation, and ATP can form via the chemiosmotic mechanism. This mechanism is based on the electrogenic transfer of an ion ( $H^+$  or  $Na^+$ ) across the cytoplasmic membrane, resulting in the generation of an ion-motive force that, in its turn, drives ATP synthesis that involves ATP synthase. From the data of Table 1 it is evident that the methylene-THF reductase reaction (reaction 5) and acetyl-CoA formation from methyl-THF plus  $CO$  (reaction 7 and 8) are sufficiently exergonic to be coupled to electron transfer phosphorylation. This process requires the operation of an ATP synthase and membrane-dependent electron transfer accompanied by proton pumping. Cytochromes, menaquinones, and Fe-S proteins were detected in a number of acetogens [29]. It was revealed that *Moorella thermoautotrophica* obtains energy via a membrane-dependent electron transfer involving an

**Table 1.** Thermodynamics of the Wood–Ljungdahl pathway reactions

	Reaction	Enzyme	$\Delta G'_0$ (kJ/mol)
1	$\text{CO}_2 + \text{NADPH} + \text{H}^+ = \text{HCOOH} + \text{NAD(P)}^+$	Formate dehydrogenase	+21.5
2	$\text{HCOOH} + \text{THF} + \text{ATP} \longrightarrow \text{ADP} + \text{P}_{\text{in}} + \text{formyl-THF}$	10-formyl-THF synthase	−8.4
3	$\text{Formyl-THF} \longrightarrow \text{OH}^- + \text{methenyl-THF}^+$	5,10-methenyl-THF cyclohydrolase	−4.0
4	$\text{Methenyl-THF}^+ + \text{NAD(P)H} \longrightarrow \text{NAD(P)}^+ + \text{methylene-THF}$	5,10-methylene-THF dehydrogenase	−4.9
5	$\text{Methylene-THF} + 2[\text{H}] \longrightarrow \text{methyl-THF}$	5,10-methylene-THF reductase	−22.0
6	$\text{CO}_2 + 2[\text{H}] \longrightarrow [\text{CO}] + \text{H}_2\text{O}$	CO deH/AcS	+41.0
7	$[\text{Co-E}] + \text{CH}_3\text{-THF} \longrightarrow \text{THF} + [\text{CH}_3\text{-Co-E}]$	CO deH/AcS, methyltransferase	−38.0
8	$[\text{CO}] + [\text{CH}_3\text{-Co-E}] + \text{KoA} \longrightarrow [\text{Co-E}] + \text{acetyl-CoA}$	Corrinoid protein, CO deH/AcS	
9	$\text{Acetyl-CoA} + \text{P}_i \longleftrightarrow \text{acetylphosphate} + \text{CoA}$	Phosphotransacetylase	+9.0
10	$\text{Acetylphosphate} + \text{ADP} \longleftrightarrow \text{acetate} + \text{ATP}$	Acetate kinase	−13.0

Note: THF, tetrahydrofolate; CO deH/AcS, CO dehydrogenase/acetyl-CoA synthase.

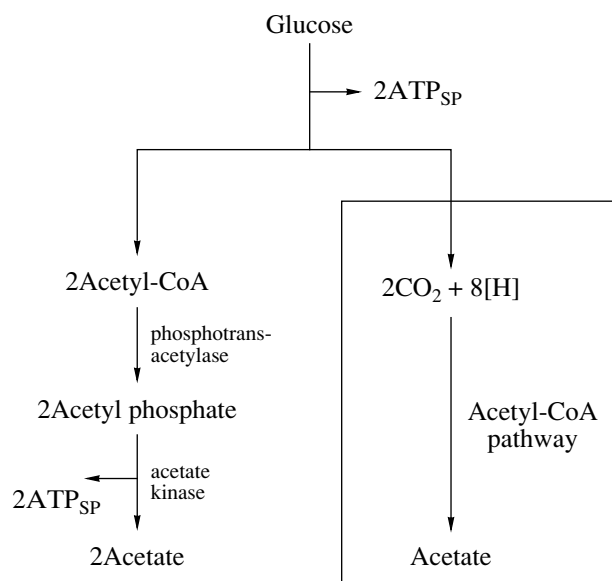
electron flow from CO, H<sub>2</sub>, or NADH via flavoprotein to two *b* type cytochromes [30]. The CO dehydrogenase, hydrogenase, and methylene-THF reductase in *M. thermoautotrophica* are membrane-bound and, therefore, can form an electrochemical proton gradient that, in its turn, can fuel ATP synthesis. A F<sub>1</sub>F<sub>0</sub> type ATPase was investigated in detail in *Moorella thermoacetica* and *Acetobacterium woodii* [31, 32].

Under heterotrophic conditions, the acetyl-CoA pathway is chiefly used as an electron sink. During glycolysis, hexose is oxidized and decarboxylated (Fig. 2) to 2 acetyl-CoA molecules and 2CO<sub>2</sub>. The 8 electrons obtained are used to reduce 2CO<sub>2</sub> to acetate via the acetyl-CoA pathway. The homoacetogenic conversion of hexose yields 4 ATP molecules at the substrate level, and additional energy is generated in chemiosmotic processes. In this system, the main goal of the pathway

is to recycle reduced electron carriers (NAD, ferredoxin, etc.) formed upon hexose oxidation [25].

**Na<sup>+</sup> requirement: Na<sup>+</sup>- and H<sup>+</sup>-dependent acetogens.** Studies concerning the energetics of the acetyl-CoA pathway revealed that growth and acetate synthesis depend in some acetogens on Na<sup>+</sup> ions. For example, acetate synthesis from CO/CO<sub>2</sub> and from H<sub>2</sub>/CO<sub>2</sub> in *Ruminococcus productus* requires Na<sup>+</sup> [28]. Na<sup>+</sup> significantly influenced the H<sub>2</sub>-dependent growth of *Thermoanaerobacter kivui*, but had no effect on its growth under heterotrophic conditions on a medium with glucose [33]. The difference in terms of the Na<sup>+</sup> requirement suggest that different mechanisms of energy conservation may operate during the auto- and organotrophic growth of *T. kivui*. Acetogenesis in *A. woodii* is strictly Na<sup>+</sup>-dependent; it is accompanied by the generation of a sodium-motive force across the cytoplasmic membrane. Recently, it has been shown that this bacterium employs a sodium cycle [27, 33]. The sodium-motive force forms at the expense of the primary sodium pump, and the resulting  $\Delta\mu_{\text{Na}}^+$  is subsequently used a driving force for flagellum rotation and ATP synthesis. Research on this bacterium made it possible to identify the Na<sup>+</sup> transfer-dependent step of the Wood–Ljungdahl pathway. Reactions 7 and 8 are the most likely candidates for this step. Demethylation (reaction 8) of the methyl-corrinoid intermediate is Na<sup>+</sup>-dependent and linked to Na<sup>+</sup> transport in methanogens, which use an acetogenesis-like methanogenesis pathway [34]. It was suggested that the transfer of a methyl group from methyl-THF to CO dehydrogenase is linked to Na<sup>+</sup> extrusion in acetogens, in an analogy to methanogens, especially as corrinoids are membrane-bound in acetogens. However, this has not yet been confirmed experimentally [27].

In contrast to the acetogens listed above, *M. thermoacetica* does not require any additional Na<sup>+</sup>, regardless of whether it grows on a glucose medium or under



**Fig. 2.** Homoacetogenic conversion of glucose to acetate [25]. SP, substrate phosphorylation.

**Table 2.** Halophilic and alkaliphilic acetogenic bacteria

Parameter	<i>Acetohalobium arabaticum</i>	<i>Natroniella acetigena</i>	<i>Natronincola histidinovorans</i>	<i>Tindallia magadiensis</i>
Family	<i>Halobacteroidaceae</i>	<i>Halobacteroidaceae</i>	<i>Clostridiaceae</i>	<i>Clostridiaceae</i>
Order	<i>Halanaerobiales</i>	<i>Halanaerobiales</i>	<i>Clostridiales</i>	<i>Clostridiales</i>
Capacity for autotrophic growth	+	–	–	–
Main substrates	Formate, lactate, trimethylamine	Lactate, ethanol	Histidine	Arginine, pyruvate
pH range (optimum)	5.6–8.4 (7.6–8.0) (neutrophile)	8.1–10.7 (9.7–10.0) (alkaliphile)	8.0–10.5 (9.4) (alkaliphile)	7.5–10.5 (8.5) (alkaliphile)
Reaction to Na <sup>+</sup>	Extreme halophile	Extreme halophile	Moderate halophile	Moderate halophile
Na <sup>+</sup> concentration range, M (optimum)	1.7–4.3 (2.56–3.08)	1.7–4.4 (2.0–2.56)	0.68–2.74 (1.37–1.7)	0.17–1.7 (0.51–1.0)

chemolithotrophic conditions with Hinoid intermediate is Na<sup>+</sup>-dependent and linked to H<sub>2</sub>/CO<sub>2</sub> or CO/CO<sub>2</sub> [28, 33]. Growth and acetogenesis are Na<sup>+</sup>-independent in *Sporomusa sphaeroides* [35].

Therefore, the Wood–Ljungdahl pathway is coupled to ATP synthesis via the chemiosmotic mechanism. However, acetogens differ in respect to the pathways they use for the formation of ion gradients across the cytoplasmic membrane [26, 33]. A number of organisms, e.g., *M. thermoacetica*, possess an electron transfer chain that contains cytochromes (despite different electron donors, the system uses only one electron acceptor, methylene-THF, which is reduced to methyl-THF) and generate a proton gradient that drives ATP synthesis using the membrane-bound H<sup>+</sup>-translocating ATP synthase. These acetogens belong to *group 1* in terms of their energetics [33]. Acetogenesis is strictly Na<sup>+</sup>-dependent in organisms that lack cytochromes, but contain membrane-bound corrinoids. Acetogenesis in *A. woodii* is coupled to the generation of a primary sodium ion gradient that is the driving force of ATP synthesis involving an F<sub>1</sub>F<sub>0</sub> type Na<sup>+</sup>-translocating ATP synthase [32, 36]. Such acetogens are classified into *group 2*, based on the peculiarities of their energetics. The properties of the F<sub>1</sub>F<sub>0</sub> ATP synthases in *M. thermoacetica* and *A. woodii* are different, reflecting the differences in the ion gradients they use to obtain chemiosmotic energy. The ATP synthase of *M. thermoacetica* is a proton pump, whereas the ATP synthase of *A. woodii* is a sodium pump.

**Halophilic acetogens.** The Microbial Communities Laboratory of the Institute of Microbiology of the Russian Academy of Sciences conducted research on the halophilic microbial community of cyanobacterial mats from the lagoons of the Arabat Spit located on the eastern coast of the Sivash Gulf [37]. A number of primary and secondary anaerobes that decompose organic substances were isolated, including the first halophilic acetogen (*Acetohalobium arabaticum*) to be identified [38]. The bacterium is a gram-negative slightly curved rod, motile (subterminal flagellation). *A. arabaticum* belongs to the order *Halanaerobiales*. It is an extreme

halophile, because it grows within a salinity range of 10 to 25% of NaCl with an optimum at 15–18% (Table 2). The optimum temperature for its growth is 38–40°C, and the maximum temperature is 47°C. The pH optimum is 7.6–8.0, and growth occurs between pH 5.6 and 8.4. *A. arabaticum* is capable of pursuing chemolithotrophic, methylotrophic, and organotrophic nutritional strategies, utilizing hydrogen, formate, lactate, aspartate, histidine, betaine, trimethylamine, and other energy substrates. *A. arabaticum* should be regarded as a unique system for biochemical studies, because it combines extreme halophily, acetogenesis as an energy-producing mechanism, and the capacity to use various nutritional strategies.

**Alkaliphilic acetogens.** Investigating the anaerobic pathway of degrading organic substances in soda lakes [2, 4, 9] enabled us to describe a number of new genera, whose representatives are involved, *inter alia*, in acetogenesis. This gave us a chance to study the alkaliphilic acetogens *Natroniella acetigena*, *Natronincola histidinovorans*, and *Tindallia magadiensis* [39–41] (Table 2). All these organisms cannot grow chemolithotrophically on H<sub>2</sub> + CO<sub>2</sub> or CO + CO<sub>2</sub> mixtures, and the range of trophic substrates they use are limited, mainly including the products of primary anaerobes. Of special interest in terms of energetics is that the organisms combine acetogenesis with alkaliphily and, therefore, should cope with the problems caused by growing at extremely high Na<sup>+</sup> contents and at pH values above 9 (and, accordingly, at low proton concentrations).

## DESCRIPTION OF ACETOHALOBIUM ARABATICUM

**Na<sup>+</sup> requirement.** Physiological and biochemical studies with *A. arabaticum* revealed its obligatory dependence on sodium ions [42]. Potassium or lithium ions equilibrated in terms of their molarity failed to replace sodium ions. Nevertheless, lithium can partially substitute sodium. The minimum NaCl amount required for the growth of *A. arabaticum* decreases from 10% (1.71 M) in a routine complex medium to 9%

(1.54 M) in the presence of lithium sulfate. Chloride has an advantage over sulfate with respect to the growth of *A. arabaticum*: prerequisite for its growth with sodium sulfate and lithium sulfate is the presence of at least 1.54 M NaCl (9%).

**Hydrogenase.** Electrons flow from hydrogen to carbon dioxide via a membrane-bound respiratory chain in homoacetogenic bacteria that grow on the  $H_2 + CO_2$  mixture. Hydrogene oxidation is catalyzed by *hydrogenase*. The hydrogenase of *A. arabaticum* was investigated by us [43]. Fractionation of *A. arabaticum* cells revealed that the organism contains a periplasmic hydrogenase, in addition to the cytoplasmic enzyme, which is peculiar to acetogenic bacteria. Over 30% of hydrogenase activity is attributed to the periplasm, while about 70% to the cytoplasm. Periplasmic hydrogenase was detected by us for the first time. This finding testifies to a specific adaptation of the halophilic *A. arabaticum* to low levels of dissolved hydrogen in hypersaline bodies of water. This is also consistent with the high enzyme activity in whole cells (0.56  $\mu\text{mol}/(\text{min mg of protein})$ ). The cytoplasmic and the periplasmic fraction of *A. arabaticum* catalyzed the reduction of an artificial electron carrier, benzylviologen, by hydrogen at a rate of 1.4–1.9 and 0.15  $\mu\text{mol}/(\text{min mg of protein})$ , respectively. The rate of benzylviologen reduction by hydrogen depended on substrate concentrations according to the Michaelis–Menten equation. The biochemical and bioenergetic functions of hydrogenases vary depending on the physiology of the bacterium involved. As was shown for sulfate-reducing bacteria of the genus *Desulfovibrio* [44], a prerequisite for the direct involvement of molecular hydrogen in the generation of the proton gradient is the availability of several hydrogenases located in different parts of the cell. Protons are reduced by a hydrogenase in the cytoplasm, and the resulting molecular hydrogen diffuses across the cytoplasmic membrane to the periplasm. Thereupon, one or several hydrogenase(s) oxidize the hydrogen to protons and electrons; the protons are utilized for ATP synthesis via reversible ATPase, and the electrons move across the cytoplasmic membrane and reduce endo- or exogenous electron acceptors. The net result of this process is the transfer of two or more protons across the cytoplasmic membrane; the process does not directly involve membrane-bound reductases or proton pumps. In an analogy to sulfate reducers, we suggest that the periplasmic hydrogenase in *A. arabaticum* contributes to the formation of the proton gradient.

**Electron carriers.** The physiological electron acceptor for the hydrogenase of *A. arabaticum* is unknown. Hydrogenase fails to reduce physiological electron carriers such as NAD (0.8 mM), NADP (1 mM), FAD (1 mM), and cytochrome *c* (1 mM). Hydrogenase-catalyzed reduction of viologen dyes in cell-free preparations is not suppressed by antimycin A (5  $\mu\text{g}/\text{mol}$  of protein), which inhibits the electron transfer chain at a site between NADH and flavin. Appar-

ently, NADH and cytochromes are not involved in the electron transfer from activated hydrogen to the terminal acceptor. Cytochromes and quinones were not detected in the electron transfer chain of *A. arabaticum* in studies using standard methods [42]. It was established that the cells of this bacterium contain flavoprotein whose  $R_f$  values differ from those of FAD, FMN, riboflavin, and lumiflavin. *A. arabaticum* also contains folates and corrinoid compounds. The corrinoids of acetogenic bacteria are important cofactors that participate in a number of redox reactions and are involved in the formation of a C–C bond during acetate synthesis via the acetyl-CoA pathway of carbon dioxide fixation. Research on the biosynthesis of corrinoids and their precursors in *A. arabaticum* revealed that its cells contain factor III, a heterocyclic nucleotide-containing corrinoid. The extracellular corrinoid uroporphyrine I and methylated uroporphyrine derivatives were also detected [45, 46].

Not all components of the electron transfer chain of *A. arabaticum* have been investigated. The organism may still contain unknown electron carriers. In contrast to the neutrophilic acetogen *Moorella thermoacetica*, *A. arabaticum* has no cytochromes and no quinones; it synthesizes corrinoids on all tested substrates. Therefore, this organism should be placed in group two (with *Acetobacterium woodii* and *Ruminococcus productus* as model organisms) based on the peculiarities of its energy metabolism [33].

**Osmoadaptation.** All tested representatives of the order *Halanaerobiales*, including *A. arabaticum*, use the “salt inside” osmoadaptation strategy that implies high intracellular  $Na^+$ ,  $K^+$ , and  $Cl^-$  concentrations and a lack of organic osmoregulators [5, 8, 47]. High salt concentrations necessitate an additional adaptation of the whole enzyme system. The enzymes of halophilic bacteria that use the “salt inside” osmoadaptation strategy are highly resistant to salts. Studies on some of the key metabolic enzymes of *A. arabaticum* revealed that they are extremely salt-tolerant (Table 3). For instance, the cytoplasmic hydrogenase is stable in concentrated salt solutions and, moreover, stimulated by these concentrations (up to the saturation level) [43]. The intracellular catabolic enzymes CO dehydrogenase, glutamate dehydrogenase, and lactate dehydrogenase are also activated by saturating sodium and potassium concentrations.

**Energy metabolism.** Research on the energy metabolism of *A. arabaticum* using metabolic inhibitors revealed that the energy-conserving mechanisms employed by this bacterium during acetogenesis vary depending on the energy-supplying substrate (Table 4) [42, 48, 49].  $H_2$  and CO oxidation in this organism is coupled to ATP synthesis via electron transfer phosphorylation based on a chemiosmotic mechanism that is linked to acetogenesis and involves an  $F_1F_0$  type ATP synthase. This is consistent with data obtained with the neutrophilic acetogens *A. woodii*, *M. thermoacetica*, and *M. thermoautotrophica* [29, 33]. ATP is predomi-

**Table 3.** Enzymes of halophilic and alkaliphilic acetogens

Microorganism	Enzyme	Activity range	Optimum concentration
<i>A. arabaticum</i>	Hydrogenase	0–4.8 M NaCl, LiCl, 0–3.6 KCl	Saturating concentration of NaCl and KCl; 1 M LiCl
<i>N. acetigena</i>	CO dehydrogenase	0–4.8 M NaCl	1 M NaCl
	CO dehydrogenase	0–4.1 M NaCl, 0–0.95 M NaHCO <sub>3</sub>	0.7 M NaCl, 0–0.7 M NaHCO <sub>3</sub>
<i>Natr. histidinovorans</i>	CO dehydrogenase	0–4.1 M NaCl, 0–1.2 M NaHCO <sub>3</sub>	0–4.1 M NaCl, without NaHCO <sub>3</sub>
<i>T. magadiensis</i>	Hydrogenase	0–4.3 M NaCl, 0–1.2 M NaHCO <sub>3</sub>	without NaCl, 0.24 M NaHCO <sub>3</sub>
	CO dehydrogenase	0–4.3 M NaCl, 0–1.2 M NaHCO <sub>3</sub>	without NaCl, 0.25 M NaHCO <sub>3</sub>

**Table 4.** Energy metabolism of halophilic and alkaliphilic acetogens

Microorganism	Substrate	Energy accumulation mechanism	ATPase type	Na <sup>+</sup> /H <sup>+</sup> -antiport	ΔpH	ΔpNa
<i>A. arabaticum</i>	H <sub>2</sub> + CO <sub>2</sub>	ETP	F <sub>1</sub> F <sub>0</sub>	+	n. d.	+
	CO + CO <sub>2</sub> + N <sub>2</sub>	ETP				
	Formate	SP				
	Trimethylamine	SP + ETP				
<i>N. acetigena</i>	Lactate	SP	H <sup>+</sup> -F <sub>1</sub> F <sub>0</sub>	+	+	+
	Ethanol	ETP				
	Lactate	ETP				
<i>Natr. histidinovorans</i>	Histidine	ETP	F <sub>1</sub> F <sub>0</sub> + E <sub>1</sub> E <sub>2</sub>	+	n. d.	+
<i>T. magadiensis</i>	Arginine	ETP	H <sup>+</sup> -F <sub>1</sub> F <sub>0</sub>	+	+	+
	Pyruvate	SP + ETP				

Note: ETP, electron transfer phosphorylation; SP, substrate phosphorylation; n. d., no data.

nantly synthesized at the substrate phosphorylation level during organotrophic growth on formate, trimethylamine, and lactate, although electron transfer phosphorylation is also involved. Based on the thermodynamic data obtained with *A. arabaticum* [50], it is more advantageous to form acetate from formate than from lactate in terms of energetics. Lactate formation in *A. arabaticum* involves a NAD-dependent lactate dehydrogenase [43]. Utilization of all energy-supplying substrates by *A. arabaticum* depends on ΔpNa, which is consistent with the inhibitory effect produced by monensin, a sodium ionophore. As for the Na<sup>+</sup> gradient-dependent ATP synthesis, two options have been suggested. The Na<sup>+</sup> gradient can be converted into a proton gradient that can drive ATP synthesis via an H<sup>+</sup> ATP synthase. This system should involve an Na<sup>+</sup>/H<sup>+</sup> antiport process. Alternatively, the Na<sup>+</sup> gradient can be directly used by an Na<sup>+</sup>-dependent ATP synthase.

### ALKALIPHILIC ACETOGENS

**Osmoadaptation.** Haloalkaliphilic acetogens use various strategies to cope with osmotic stress caused by high salt concentrations. The alkaliphilic haloanaerobe *Natroniella acetigena* (order *Halanaerobiales*) main-

tains very high intracellular Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>−</sup> concentrations, which increase further upon increasing NaCl amounts in the medium. This enables the organism to equilibrate the internal osmotic pressure with that of the highly mineralized medium (Table 5) [49, 51]. The intracellular Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>−</sup> concentrations in *N. acetigena* are close to those in other haloanaerobes [8]. The intracellular chloride concentration is usually maintained at a level that is equivalent to the chloride concentration in the environment, while the sodium concentration inside the cell is slightly below, or almost equal to that in the medium. The intracellular potassium concentration is several times higher than the potassium concentration in the environment [6, 8]. It is generally accepted that membrane potential (with the negative charge inside the cell) is the driving force for K<sup>+</sup> accumulation [8]. K<sup>+</sup> ions enter the cell, while Na<sup>+</sup> ions extrude at the expense of the Na<sup>+</sup>/H<sup>+</sup> antiporter, and the whole system remains electroneutral. The intracellular Na<sup>+</sup> and K<sup>+</sup> concentrations in *N. acetigena* are sufficiently high, and the intra- and extracellular Cl<sup>−</sup> concentrations are equilibrated. Such high intracellular salt concentrations imply a lack of organic osmoregulators [51] that is characteristic of the representatives of the order *Halanaerobiales* that were tested earlier [5, 8]. In this system, K<sup>+</sup> and plausibly also Cl<sup>−</sup> perform an

**Table 5.** Intracellular and extracellular Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>−</sup> concentrations in *N. acetigena* and *T. magadiensis*

Microorganism and cultivation conditions	Na <sup>+</sup> outside (M)	Na <sup>+</sup> inside (M)	K <sup>+</sup> outside (M)	K <sup>+</sup> inside (M)	Cl <sup>−</sup> outside (M)	Cl <sup>−</sup> inside (M)
<i>N. acetigena</i> ; 1% NaCl, 1.91 M Na <sup>+</sup> <sub>(total)</sub>	2.07	0.91	0.013	0.83	0.18	0.29
<i>N. acetigena</i> ; 5% NaCl, 2.59 M Na <sup>+</sup> <sub>(total)</sub>	2.78	1.98	0.013	0.94	0.86	0.89
<i>T. magadiensis</i> ; without NaCl, 0.48 M Na <sup>+</sup> <sub>(total)</sub>	0.49	0.40	0.004	0.17	0.012	0.009
<i>T. magadiensis</i> ; without NaCl, 1.03 M Na <sup>+</sup> <sub>(total)</sub>	1.15	0.65	0.004	0.22	0.015	0.014

Note: Outside, ion concentration in the supernatant after precipitating the cells; Inside, intracellular ion concentration.

osmoprotective function, which is more advantageous than the synthesis of organic osmoregulators at extremely high external salt concentrations [5]. However, adaptation to high intracellular salt concentrations requires alterations in the whole enzyme system of the cell. The enzymes of such organisms display maximum activity in the presence of salts and, moreover, are inactivated in their absence. For example, the CO dehydrogenase of *N. acetigena* is a typical halophilic enzyme whose activity is stimulated by NaCl (Table 3).

In contrast to *N. acetigena*, *Tindallia magadiensis*, another haloalkaliphilic acetogen, maintains moderate intracellular Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>−</sup> concentrations that vary depending on the external NaCl concentration (Detkova, unpublished, see [49]). If the medium contains trace amounts of Cl<sup>−</sup> ions, their intracellular concentration is also insignificant (Table 5). The ion concentrations in the intracellular space are too low to equilibrate the external osmotic pressure in a highly saline medium. This points to an ancillary role of K<sup>+</sup> ions in the osmoadaptation system of *T. magadiensis*. Our preliminary data indicate that *T. magadiensis* cells contain glycine–betaine. This compound is a widespread osmoregulator in halophiles. The tested *T. magadiensis* enzymes are salt-tolerant, although they differ in sensitivity to salts. For instance, the maximum hydrogenase and CO dehydrogenase activities in *T. magadiensis* are attained without NaCl, but the enzymes retain a significant part of their activities at saturating concentrations of NaCl. This is consistent with the osmoadaptation strategy (the “compatible osmoregulators” strategy) selected by *T. magadiensis*. The CO dehydrogenase of *T. magadiensis* displays the maximum activity with 0.25 M NaHCO<sub>3</sub>, whereas hydrogenase activity only slightly varies depending on the NaHCO<sub>3</sub> concentration within the 0–1.2 M range (Table 3).

**Energy metabolism.** Research on the energy metabolism of *Natroniella acetigena* and *Natronincola histidinovorans* using metabolism inhibitors and ionophores demonstrated that the oxidation of energy-supplying substrates (lactate, ethanol, histidine) is coupled to ATP generation via electron transfer phosphorylation based on a chemiosmotic mechanism that involves an ATPase [49, 52–54] (Table 4). *N. acetigena* is likely to contain an F<sub>1</sub>F<sub>0</sub> type ATPase, while *N. histidinovorans* may contain both an F<sub>1</sub>F<sub>0</sub> and E<sub>1</sub>E<sub>2</sub> type ATPases [52].

The moderate alkaliphile *T. magadiensis* displayed a somewhat different pattern [49, 55]. Arginine fermentation in this bacterium is coupled to ATP generation via electron transfer phosphorylation based on a chemiosmotic mechanism that involves an F<sub>1</sub>F<sub>0</sub> type ATPase and is linked to acetogenesis. Using arginine as a substrate implies its involvement in the ornithine cycle and subsequent conversion to carbamoyl phosphate with the formation of CO<sub>2</sub> and NH<sub>3</sub> catalyzed by carbamate kinase. This reaction is coupled to ATP formation from ADP and P<sub>in</sub> involving an F<sub>1</sub>F<sub>0</sub> type ATP synthase [56]. CO<sub>2</sub> is there upon incorporated in the acetyl-CoA pathway, resulting in the formation of acetate as the terminal product. If *T. magadiensis* was cultivated on pyruvate, ATP formation was predominantly based on substrate level phosphorylation, although we can also suggest the involvement of oxidative phosphorylation in energy accumulation during acetogenesis (Table 4).

#### Ion gradients across the cytoplasmic membrane.

Studies with cell suspensions of *N. acetigena* revealed that a proton gradient is the driving force of ATP synthesis. The method used in these studies was based on determining the ATP synthesis in cells in response to an artificial pH gradient. The publications [54, 57, 58] present evidence that *N. acetigena* contains an ATPase that uses H<sup>+</sup> as the coupling ion: instantaneous ATP synthesis was observed that depended on the artificial ΔpH as the driving force. A 100-fold increase in the H<sup>+</sup> concentration resulted in a 2.5-fold increase in the intracellular ATP content, compared to the endogenous level in the control system. ATP synthesis is the physiological function of the H<sup>+</sup> ATPase coupled to the proton pump. Hence, oxidative phosphorylation depends on proton translocation in this bacterium. The presence of an Na<sup>+</sup>-translocating ATPase in *N. acetigena* cannot be ruled out, but research on the effects of an artificial sodium gradient is unfeasible, because cell lysis occurs in Na<sup>+</sup>-lacking buffer mixtures.

Growth and acetogenesis require an electrochemical proton potential and a pH gradient in *N. acetigena*. Cell deenergization by protonophores inhibits Na<sup>+</sup>/H<sup>+</sup> antiport, removing the osmotic barrier of the cytoplasmic membrane, which results in cytoplasm alkalization and cell death. The bacterial culture also depends on the Na<sup>+</sup> potential, which is demonstrated by the effect of the sodium ionophore monensin [53]. This ionophoric



antibiotic completely suppressed growth and acetogenesis in *N. acetigena*. Hence, the physiology of *N. acetigena* is characterized by major energy investments aimed at maintaining the intracellular alkalinity level in a carbonate medium and based on a combination of the proton and sodium cycles. The above facts indicate that, unlike neutrophilic acetogens, the extreme haloalkaliphile *N. acetigena* requires the proton cycle linked to oxidative phosphorylation and the  $F_1F_0$  ATPase to transfer protons into the cell and maintain pH homeostasis. An analogous function of the proton cycle was established in aerobic alkaliphilic bacilli [22]. The sodium potential is generated via electroneutral or electrogenic  $Na^+/H^+$  antiport. The proton and sodium cycle are based on electrogenic  $Na^+/H^+$  antiport in aerobic extremely alkaliphilic bacilli [17].

It was established that *N. acetigena* uses a coherent system that provides for membrane energization at the expense of the sodium gradient, ATP synthesis involving an  $H^+$ -dependent ATPase, and the operation of a  $Na^+/H^+$  antiport system that maintains homeostasis by forming  $\Delta pH$ . Therefore,  $\Delta pH$  is directly used for accumulating energy in the form of ATP by the  $H^+$ -dependent ATPase of this organism, and a similar pattern is characteristic of the representatives of group one of neutrophilic acetogens (*M. thermoacetica*, *M. thermoautotrophica*, and *S. sphæroides*) [33]. The issue of proton-dependent oxidative phosphorylation at high pH values has not yet been settled. Non-stoichiometric mechanisms and a high  $H^+/ATP$  stoichiometry were postulated to account for proton-dependent ATP synthesis in aerobic alkaliphiles [7, 59]. In the haloalkaliphile *N. acetigena* this process can form part of its osmoadaptation strategy. Accumulating a high intracellular  $Na^+$  concentration precludes the formation of a significant sodium gradient across the cytoplasmic membrane:

$\Delta pNa = Na^+_{out}/Na^+_{in} = 2.3$  (Table 5). This  $\Delta pNa$  is insufficient for ATP synthesis. For the sake of comparison, note that  $\Delta pNa = 32$  [60] in the neutrophilic acetogen *A. woodii*.

The development of *Natr. histidinovorans* and *T. magadiensis* depends on the proton potential to a lesser extent than that of *N. acetigena*, because protomorphores fail to inhibit their growth and acetogenesis [52, 55]. In these systems, the sodium cycle possibly plays a key role in maintaining the intracellular pH level and transferring substances into the cell, which is confirmed by the inhibitory effect of monensin. The results of preliminary inhibitor studies suggest that an  $H^+$ -dependent  $F_1F_0$  type ATPase carries out ATP synthesis in *T. magadiensis*, in an analogy to *N. acetigena*. Apparently,  $\Delta pNa$  converts to  $\Delta pH$  in an  $Na^+/H^+$  antiport-involving process coupled to ATP synthesis via the  $H^+$ -ATPase.

Thus, research on the energy metabolism of halophilic and alkaliphilic bacteria revealed various strategies of adaptation to high salinity and high pH values. An inhibitor assay demonstrated that halo- and alkaliphilic acetogens use various energy-storing mechanisms including electron transfer or substrate phosphorylation and transmembrane  $Na^+$  and  $H^+$  gradients,

depending on the energy-supplying substrate and the metabolic strategy. The  $Na^+/H^+$  antiport system is a universal mechanism of generating ion gradients and maintaining pH homeostasis in alkaliphiles.

The haloanaerobes *A. arabaticum* and *N. acetigena* use the "salt inside" osmoadaptation strategy that implicates accumulation of high intracellular  $Na^+$ ,  $K^+$ , and  $Cl^-$  gradients and a lack of organic osmoregulators. In contrast, the haloalkaliphile *T. magadiensis* employs the "compatible osmoregulators" strategy.

The lithotrophic growth of the extremely halophilic eubacterium *A. arabaticum* is coupled to ATP synthesis via electron transfer phosphorylation based on the chemiosmotic mechanism involving an  $F_1F_0$  type ATPase. This is consistent with the data obtained with the neutrophilic acetogens *T. kivui*, *A. woodii*, *M. thermoacetica*, and *M. thermoautotrophica*. *A. arabaticum* performs the reduction of  $CO_2$  to acetate via the Wood-Ljungdahl acetyl-CoA pathway involving hydrogenase, CO dehydrogenase, ATP synthase, the  $Na^+/H^+$  antiportsystems, corrinoid proteins, folates, and flavins. During organotrophic growth, ATP is synthesized at the substrate phosphorylation level. Research on the energy metabolism of haloalkaliphilic acetogenic bacteria revealed that the oxidation of organic energy-supplying substrates by *Natroniella acetigena* (lactate and ethanol) and *Natronincola histidinivorans* (histidine) is coupled to ATP generation via electron transfer phosphorylation based on an ATPase-involving chemiosmotic mechanism. This distinguishes the bacteria from the neutrophilic haloanaerobe *A. arabaticum*. Possibly, the ATP formed via substrate phosphorylation in the alkaliphilic *N. acetigena* is spent to maintain the proton gradient. Hence, no substrate phosphorylation-dependent ATP accumulation can occur.

*N. acetigena* and *Natr. histidinovorans* display considerably different strategies of adaptation to extreme environmental conditions. The haloanaerobe *N. acetigena* belongs to a self-contained phylogenetic branch of the order *Haloanaerobiales*, and its adaptation to an alkaline medium is based on an active bioenergetic mechanism. The bacterium should be considered a halophile adapted to alkaline milieu. In contrast, *Natr. histidinovorans*, a representative of the phylogenetic branch of low G+C *Clostridia*, turned out to be a true alkaliphile, and the inhibition of energy metabolism does not cause its destruction. The energy metabolism of the moderately halophilic acetogenic bacterium *T. magadiensis* is based both on the sodium cycle and proton gradient. The energy accumulation mechanisms used by the bacterium in acetogenesis vary depending on the energy-supplying substrate utilized by it.

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