Microbiology, Vol. 71, No. 2, 2002, pp. 123–132. Translated from Mikrobiologiya, Vol. 71, No. 2, 2002, pp. 149–159.

REVIEW PAPER

The Biology and Osmoadaptation of Haloalkaliphilic Methanotrophs

Yu. A. Trotsenko and V. N. Khmelenina

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

Received April 4, 2001; in final form, June 26, 2001

Abstract—There is increasing evidence for the presence and activity of methanotrophic bacteria in saline and alkaline aquatic environments located in different ecogeographical regions. Alkalitolerant halophilic and alkaliphilic halotolerant methanotrophs of type I were found to be able to utilize methane and methanol, to oxidize ammonium ions, and to transform various organic compounds in a wide range of water salinities (up to 12% NaCl) and pH values (from 5 to 11). The ecophysiological importance of methanotrophs in microbial communities inhabiting saline and alkaline aquatic environments is due to their involvement in the global cycles of methane and major bioelements (C, N, and S). Specific cyto- and biochemical properties of haloalkaliphilic methanotrophs—the synthesis of osmoprotectants (ectoine, 5-oxoproline, and sucrose), the accumulation of potassium ions, the formation of glycoprotein S-layers on the outer surface of their cell walls, and the modification of the chemical composition of their membranes—allow them to adapt to highly saline and alkaline habitats. Due to their specific properties, haloalkaliphilic methanotrophs may be of use in modern biotechnology.

Key words: halophilic and alkaliphilic methanotrophs, salt and soda lakes, osmoadaptation, osmoprotectants, S-layers.

INTRODUCTION

Historical background. Methane-utilizing bacteria, or methanotrophs, are widespread in nature and greatly contribute to the global biogeochemical cycles of carbon, nitrogen, and other bioelements. By consuming methane, methanotrophs reduce the threat of global warming through the greenhouse effect. Many methanotrophs are able to oxidize a wide range of aliphatic and aromatic compounds and, therefore, may be involved in bioremediation of the environment from these toxic pollutants. Until recently, most collection cultures of methanotrophs have been represented by neutrophilic strains, which show optimal growth at pH values between 6.5 and 7.5 and salinities not exceeding 0.5% NaCl [1–3]. The only exception was marine methanotrophs, which can grow at salinities of up to 3% NaCl [4].

In 1949, Hutton and Zobell reported on the isolation of methanotrophs from marine sediments [5], but it was not until 40 years later that Lidstrom presented the first taxonomic description of marine methanotrophs [6]. Further studies of marine ecosystems confirmed the presence and activity of methanotrophic bacteria there [7, 8]. Malashenko *et al.* described methanotrophs that can tolerate up to 9% NaCl, which were isolated from salt lakes, although those methanotrophs were not obtained in pure cultures [9, 10]. Until now, there was no evidence that methanotrophs are present in alkaline ecosystems.

In spite of the fact that soda and salt lakes comprise up to 80% of inland bodies of water in certain regions [11], they are far from being well studied with respect to their microbiology. In his pioneering work, Issatchenko emphasized the diversity of microbiological processes in soda lakes [12]. In recent years, there has been an increasing interest by researchers in the study of athalassic lakes located in the African Rift System, Central Asia, Southeast Siberia, and North America. The complex study of these ecosystems allowed different physiological groups of (halo)alkaliphilic microorganisms to be established [13–18]. Hypersaline lagoons and athalassic soda lakes are believed to be relic biotopes, where diverse terrestrial microbiota have evolved [19].

The microflora of saline and alkaline aquatic environments have been described in a number of review papers [11, 20–23]. The conditions of high mineralization and/or alkalinity of such environments regulate their prokaryotic communities, which include the major trophic groups of microorganisms. The primary producers in these environments are cyanobacteria and photosynthetic purple bacteria. The final link in the food chain of such ecosystems is made up of heterotrophic bacteria belonging to different physiological and taxonomic groups [20, 22, 24]. The microbial communities of thalassic and continental aquatic environments are essentially similar in their trophic structure. The major functional groups of microorganisms commonly belong to different genera [16, 20].

0026-2617/02/7102-0123$27.00

By the time our investigations were started, all specific reactions of the carbon cycle in saline and alkaline ecosystems, from the fixation of carbon dioxide to the formation of methane (one of the major end products of decomposing organic matter in anoxic environments), had been studied. At the same time, little was known about the methane sink in such ecosystems. The oxidation of methane by methanotrophs seemed questionable because of the low solubility of this gas and because the gas chromatographic and microscopic analyses of samples taken from hypersaline microbial mats failed to detect methanotrophic bacteria and methane consumption [25, 26]. We were the first to show a gradual consumption of (^{14}C) methane in the Crimean brine lakes with water salinities from 8 to 30% [27]. The rate of methane consumption was found to be maximal at salinities between 10 and 12%. Our further efforts were aimed at demonstrating the existence of methanotrophs in hypersaline and/or hyperalkaline environments and at studying their biology. For this purpose, it was necessary to develop more specific and sensitive methods for detecting, isolating, and investigating (halo)alkaliphilic methanotrophs.

Detection and isolation of haloalkaliphilic methanotrophs. Using ^{14}C-labeled methane, we revealed methane consumption in bottom sediment and water samples taken from salt and soda lakes in different ecogeographical regions. The maximum rates of methane oxidation were observed in sediment samples taken in the severely alkaline but slightly mineralized lakes of Tuva and Buryatia. As water salinity rose to 15%, the rates of $^{14}CH_4$ assimilation by bacterial cells and the incorporation of ^{14}C into their exometabolites increased. However, a further increase in water salinity led to a decrease in these rates to almost zero. The decrease in methane assimilation was especially profound in the case of some hypersaline habitats of Ukraine and Mongolia [27–29]. A high rate of methane oxidation was observed within a wide range of pH values (from 5 to 11), with two peaks at pH 5.8–6.0 and at 8.2–9.4. Presumably, methanotrophs have adapted to considerable changes in environmental pH due to seasonal changes in water level. In some soda lakes of southeast Transbaikal, the intensity of methane oxidation was twofold lower than the intensity of methane formation [30], indicating that the methanotrophs of such habitats can potentially utilize all of the biogenic methane produced there. It should, however, be noted that the rates of methane formation and oxidation in these studies were measured under conditions where the oxygen supply differed. Therefore, the real scale of methanogenesis and methane oxidation in situ remains to be evaluated.

The presence of methanotrophs in virtually all the samples under study was confirmed by the polymerase chain reaction (PCR) technique using specific genetic probes (primers) to detect genes encoding the α-subunits of methanol dehydrogenase (*mxaF*) and soluble and particulate methane monooxygenases (*mmoX* and *pmoA*, respectively) [31]. It should be noted that the primers that were used to detect methanotrophs in saline and alkaline ecosystems allow the detection of marine methanotrophs only after their isolation in enrichment cultures [32].

The microscopic examination of enrichment cultures obtained from most sediment and water samples in a methane–oxygen atmosphere showed that they represent associations of heterotrophic and methanotrophic bacteria, which could not be separated without destroying their methanotrophic component. The content of methanotrophs in enrichment cultures could be augmented by using special approaches, such as aseptic filtration and centrifugation, which allow larger and heavier methanotrophic cells to be separated from smaller and lighter heterotrophic satellites. In some cases, the addition of the culture liquid of heterotrophic satellites exerted a beneficial effect on the growth of methanotrophs, presumably due to the fact that the heterotrophs are able to synthesize growth factors and osmoprotectants essential to the methanotrophs. The repeated transfers of subcultures, with the period between the transfers being gradually shortened, led to the isolation of strains that do not require any exogenous growth factors and can grow in pure cultures. Almost all sediment and water samples taken from soda and salt lakes contained protozoans grazing on methanotrophs [4]. For this reason, the addition of 20 μg/ml cycloheximide was a necessary condition for the successful isolation of methanotrophs. Together with the serial dilution technique, all the aforementioned approaches allowed us to isolate seven haloalkaliphilic methanotrophs in pure cultures [33–36], which formed the basis of the first collection of such bacteria.

THE PROPERTIES AND TAXONOMY OF HALOALKALIPHILIC METHANOTROPHS

Morphology and physiology. New methanotrophic isolates represent large nonpigmented rod-shaped cells 1.5×3.0 μm in size with singular or multiple polar flagella. The cell walls of methanotrophs resemble those of gram-negative bacteria but contain, on their outer surface, unusual structured layers (called S-layers) with linear (*p*2) or hexagonal (*p*6) symmetry. Intracytoplasmic membranes (ICMs) look like stacks of vesicular disks, which is the typical appearance of such membranes in type I methanotrophs. The methanotrophs are able to utilize methane and methanol as sources of carbon and energy and nitrate and ammonium salts as sources of nitrogen. Even at a concentration as high as 7%, methanol is not inhibitory to the growth of the most isolates, which is uncommon for type I methanotrophs. Some of the methanotrophs can tolerate desiccation and heating at 80°C for 20 min, which makes their maintenance easier. The high resistance of these methanotrophs to heating and desiccation probably evolved in response to drastic seasonal changes in ambient temperature (from 40°C in summer to –40°C in winter),

which can lead to the complete drying out or freezing of salt and soda lakes in Mongolia and southeastern Siberia. A unique feature of the new methanotrophic isolates is their ability to grow at high water salinities (8–12% NaCl) and pH values (up to 11).

Carbon and nitrogen metabolism. All of the known halophilic and alkalitolerant methanotrophs assimilate methane through the ribulose monophosphate (RuMP) pathway, have the incomplete Krebs cycle (because of the absence of α-ketoglutarate dehydrogenase and the glyoxylate cycle), and possess only one, $NADP^+$-dependent, isocitrate dehydrogenase. Like other type I methanotrophs, (halo)alkaliphilic methanotrophs utilize phosphosugars via three different pathways (glycolytic, pentose phosphate, and Entner–Doudoroff). The glycolytic pathway is reduced because of the absence of pyruvate kinase, so that phosphoenolpyruvate is the end product of glycolysis. The phosphorylation of fructose-6-phosphate requires pyrophosphate (PP_i) rather than ATP as the source of a phosphate group.

Although NaCl stimulates the oxidation of CH_4 by haloalkaliphilic methanotrophs [37], cytoplasmic formate dehydrogenase and hexulose-6-phosphate synthase are inhibited by 0.1 M NaCl in vitro and have pH optima at a pH of 7.5–8.0. Therefore, to provide for the maximum growth rate under alkaline conditions (at pH > 9) in the presence of NaCl, bacterial cells must control the intracellular concentrations of hydrogen, sodium, and chloride ions. The mechanisms maintaining the homeostasis of these ions in haloalkaliphilic methanotrophs are as yet poorly studied. However, the higher rates of methane assimilation by cells adapted to alkaline conditions in comparison with cells grown at neutral pH values suggest that the homeostatic regulation of intracellular pH in alkaliphilic methanotrophs is inducible [35].

The methanotrophs isolated from slightly mineralized soda lakes are characterized by a relatively high affinity for methane, as is evident from the value of the saturation constant for this substrate (K_s = 2.9 μM). It should be noted that this value of the saturation constant is specific to neutrophilic methanotroph, whereas the methanotrophs isolated from highly mineralized aquatic habitats exhibit an even higher affinity for methane (K_s = 0.9 μM). This indicates that the enzymatic apparatus of such methanotrophs is adapted to the methane deficiency caused by the low solubility of this gas in high-osmolarity media [35].

The growth of haloalkaliphilic methanotrophs in alkaline media is inhibited by concentrations of ammonium ions as low as 0.3 mM. This is likely due to alkalinization of the cytoplasm, since, at alkaline pH values, ammonium ions convert to free ammonia. The ammonia easily penetrates into the cytoplasm, where it undergoes back conversion to ammonium ions, thus decreasing the concentration of protons in the cytoplasm. The haloalkaliphilic methanotrophs studied so far were found to be able to oxidize ammonium ions to nitrite attaining their optimum at pH 9.5–10.0. Accordingly, haloalkaliphilic methanotrophs are able to implement the first phase of nitrification and, together with other microorganisms, are probably involved in the conversion of fixed nitrogen in alkaline ecosystems [29, 38]. Like other type I methanotrophs, haloalkaliphilic methanotrophs assimilate ammonium ions through the reductive amination of pyruvate and α-ketoglutarate, as well as by the glutamate cycle.

Taxonomic position. The G+C content of the DNA of the haloalkaliphilic isolates varies from 46 to 51 mol %. Based on their pheno- and genotypic characteristics, including the nucleotide sequence of 16S rDNA, the isolates were preliminarily classified as three new species of the genus *Methylomicrobium: M. alcaliphilum* (strains 5Z and 20Z), *M. modestohalophilum* (strain 10S), and *M. buryatense* (strains 4G, 5G, 6G, 7G, and 5B). Since the type species of this genus, i.e., the marine halophilic methanotroph *M. pelagicum*, is not available from the main world's collections of microorganisms, the DNA–DNA hybridization of the isolates was carried out with another representative of the genus, the neutrophilic nonhalophilic methanotroph *M. album* BG8, which has cup-shaped cell surface structures close to, but not identical with, those of the methanotrophs *M. alcaliphilum* and *M. buryatense*. The DNA–DNA hybridization levels of the haloalkaliphilic isolates with *M. album* BG8 were found to be about as low as 3%, indicating that the isolates may represent a new genus, for which we have proposed the name "*Methylohalonatronum*". According to its DNA–DNA hybridization level, the haloalkaliphilic methanotroph *Methylomicrobium kenii* AMO1, isolated from mud samples taken in Kenyan soda lakes [38], should also be ascribed to this new genus. The low degree of DNA homology (below 20%) between the neutrophilic halophilic bacterium *M. modestohalophilum* 10S, isolated from the Crimean Lake Sasyk with hypersaline (14% NaCl) and neutral (pH 7) water, *M. alcaliphilum* strains 20Z and 5Z, isolated from Tuva soda lakes, and *M. buryatense*, isolated from soda lakes in the Southern Transbaikal region, indicates that the genomes of alkaliphilic and neutrophilic methanotrophs differ considerably. At the same time, the degree of DNA homology between *M. modestohalophilum* 10S and the neutrophilic nonhalophile *M. album* BG8 turned out to be relatively high (52%).

Thus, we succeeded in isolating new taxa of haloalkaliphilic and haloalkalitolerant methanotrophs from hydrochemically different bodies of water situated in geographically distant regions. In spite of the relatively high degree of DNA–DNA homology (72–82%) between various *M. buryatense* strains, they differ phenotypically to an appreciable degree particularly with respect to their dependence on sodium ions, the optimal pH values for growth, the upper limits of salt and heat tolerance, and the size of the cup-shaped cell surface structures (S-layers). Soluble methane monooxygenase

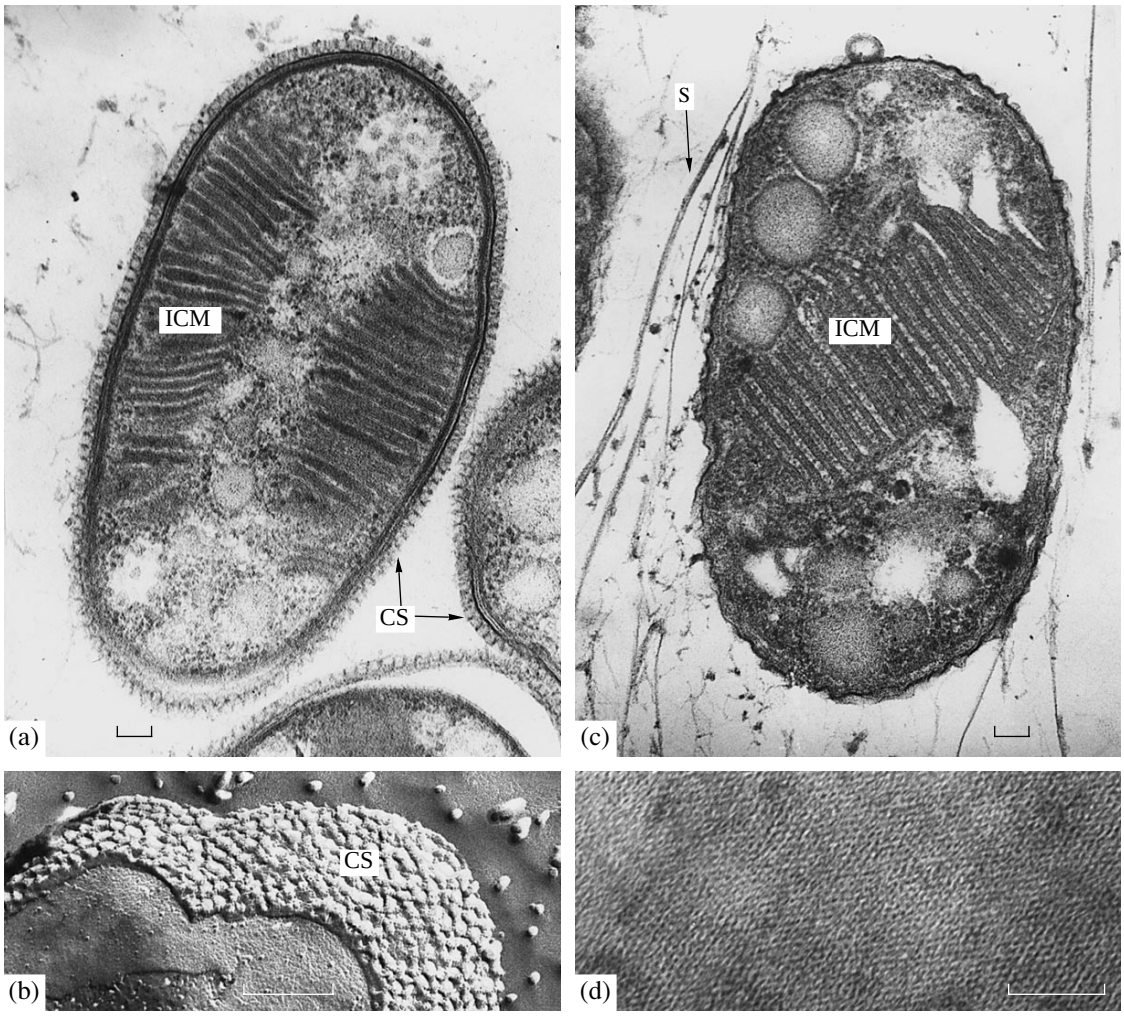

Fig. 1. Cell morphology and ultrastructure of halo- and alkaliphilic methanotrophs: (a) thin section and (b) freeze-fracture replica of *M. alcaliphilum* 20Z cells; (c) thin section and (d) S-layer with p2 symmetry of *M. modestohalophilum* 10S cells under a phase-contrast microscope. ICM, intracytoplasmic membrane; CS, cup-shaped structure; S, S-layer. Bars represent 0.1 μm.

was detected only in one *M. buryatense* strain, 5G [35]. Most of the haloalkaliphilic and haloalkalitolerant methanotrophic bacteria isolated so far are type I methanotrophs with the RuMP pathway of formaldehyde assimilation. There is only one haloalkaliphilic type II methanotroph with the serine pathway of C1-metabolism. Due to the fact that haloalkaliphilic methanotrophs strictly depend on methane and methanol, their central metabolism pathways are almost identical. At the same time, haloalkaliphilic methanotrophs differ distinctly in the physiological and biochemical properties that are directly related to the adaptation of these bacteria to their specific habitats. This makes haloalkaliphilic methanotrophs suitable for studying the general and specific mechanisms of bacterial osmoadaptation. To ascertain the diversity and structure of methanotrophic populations in saline and alkaline biotopes, further molecular ecological investigations are necessary.

MECHANISMS RESPONSIBLE FOR THE OSMOADAPTATION OF METHANOTROPHS

The ultrastructure of the cell surface of methanotrophs. As mentioned above, the cell surface of *M. modestohalophilum* 10S and *M. alcaliphilum* 20Z bacteria contains unusual structures of two types: scale-like structures with *p*2 symmetry and cup-shaped structures with *p*6 symmetry (Fig. 1). Cup-shaped structures of different height and diameter were also observed for all of the *M. buryatense* strains studied. The cell wall of *M. kenii* cells was found to contain globular electron-opaque S-layers [38]. S-layers are most clearly seen at a high magnification on the surface of cells fixed with ruthenium red, which is an indication that these layers contain polysaccharides. It should be noted that *M. alcaliphilum* 20Z cells grown on methane at pH 7.2 in the presence of NaCl, or in a medium with methanol, lack cup-shaped surface structures but possess them when grown at neutral pH values in the presence of

NaCl, or at alkaline pH values in the absence of NaCl, or under the conditions of methane deficiency [39].

The isolated and purified surface structures of *M. modestohalophilum* 10S cells subjected to electrophoresis in polyacrylamide gel showed the presence of only one protein band with a molecular mass of 27 kDa. At the same time, the surface structures of *M. alcaliphilum* 20Z exhibited several protein bands with molecular masses from 10 to 45 kDa, indicating a complex protein composition for the S-layers of this bacterium. The protein content of the surface structures of strains 10S and 20Z was 80 and 96%, respectively. Analysis of the purified cell-surface proteins of both strains showed that they are dominated by acidic and hydrophobic amino acids, whereas the sulfur-containing amino acids cysteine and methionine are present in minor amounts. The carbohydrate component of the surface structures of strain 10S is primarily composed of glucose and galactose (76 and 14%, respectively), and that of strain 20Z is made up of mainly glucose (92%).

Thus, the cell wall of most haloalkaliphilic and halotolerant methanotrophs contains various glycoprotein structures (S-layers), which represent the major line of defense for cells and may play the role of a molecular or ionic trap or of a rigid casing for the cell envelope [40, 41]. Specifically, the S-layers of haloalkaliphilic methanotrophs are supposed to be porin-like molecular traps for methane under the conditions of its low solubility at high water salinities and/or pH values. Allowing for the fact that methane solubility decreases with increasing temperature, the occurrence of S-layers with tetragonal symmetry on the cell surface of the thermotolerant methanotroph *Methylococcus capsulatus* [42] indirectly confirms the above supposition. The actual role of S-layers in the adaptation of haloalkaliphilic methanotrophs to extreme environmental conditions can be better understood in the course of further cytobiochemical and molecular genetic investigations.

Phospholipid composition. Analysis of the phospholipid composition of methanotrophic isolates showed that the major phospholipids of the neutrophilic bacterium *M. modestohalophilum* 10S are phosphatidylethanolamine and phosphatidylglycerol. In addition, the haloalkaliphilic bacterium *M. alcaliphilum* 20Z contains phosphatidylcholine and phosphatidylserine. Both methanotrophs respond to the increase in environmental salinity by raising the content of negatively charged phosphatidylglycerol and lowering the content of phosphatidylethanolamine. Such a response is specific to many gram-negative halophilic and halotolerant eubacteria [43, 44]. It is believed that negatively charged phospholipids, particularly phosphatidylglycerol, enhance the cation permeability of bacterial membranes and stabilize them by increasing their transmembrane potential. High pH values in the environment promote the accumulation of phosphatidylglycerol (and phosphatidylcholine in the case of *M. alcaliphilum* 20Z) in the membranes of haloalkaliphilic methanotrophs [35, 39]. Taking into account that phosphatidylglycerol and phosphatidylcholine are the major phospholipids of membrane bilayers, these observations suggest that further stabilization of cell membranes is necessary for the normal growth of haloalkaliphilic and haloalkalitolerant methanotrophs at high values of osmolarity and pH.

Osmoprotectants. The investigation of intracellular osmolytes provided deeper insight into the mechanisms of the osmoadaptation of methanotrophs. Proton NMR studies revealed the presence of ectoine (2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid), a heterocyclic amino acid with high water-binding capacity, in almost all of the new methanotrophic isolates [45–47]. Another osmolyte, glutamate, was detected in considerably smaller amounts. The content of glutamate in cells depends insignificantly on the salinity of the medium. The methanotrophic bacteria grown in high-salinity media can also accumulate 5-oxoproline (the cyclic form of glutamate) and, under nitrogen deficiency, sucrose [48].

The calculations based on NMR data and the intracellular water content showed that the concentration of ectoine and 5-oxoproline in *M. alcaliphilum* 20Z cells grown at a NaCl concentration of 1 M may reach 1.5 and 0.4 M, respectively. In this case, the intracellular concentration of potassium ions was found to be 0.4 M, indicating the outward concentration gradients of these ions. The equimolar concentrations of potassium ions and 5-oxoproline (or glutamate) in methanotrophic cells suggest that K^+ are counterions of these osmolytes. In general, the intracellular contents of osmolytes and potassium ions are sufficiently high to maintain osmotic equilibrium between the cytoplasm and the environment. The content of ectoine in the haloalkaliphilic strain 20Z was found to be fivefold higher than in the neutrophilic strain 10S. This observation suggests that the high value of the optimum growth pH (9.0) of *M. alcaliphilum* 20Z cells is determined by their high capacity for the synthesis of nitrogen-containing osmolytes (ectoine, glutamate, and 5-oxoproline), as a result of which these cells can tolerate the double stress of high osmolarity and pH. When the biosynthesis of nitrogen-containing osmolytes is limited, for instance, by a nitrogen deficiency in the medium, the cells can tolerate such stress by accumulating sucrose and glycogen.

Biosynthesis of osmoprotectants. Ectoine, 5-oxoproline, and sucrose are neither the growth substrates of obligate methanotrophs nor the intermediates of their central metabolism. Therefore, they are synthesized in response to osmotic stress via special pathways, which were revealed by measuring relevant enzymes in *M. alcaliphilum* 20Z cells grown at differing salinities of the medium [48]. As can be seen from the diagram presented in Fig. 2, sucrose is synthesized from intermediates of the RuMP pathway in a branch of the glycogen-synthesizing pathway. Constitutive sucrose-

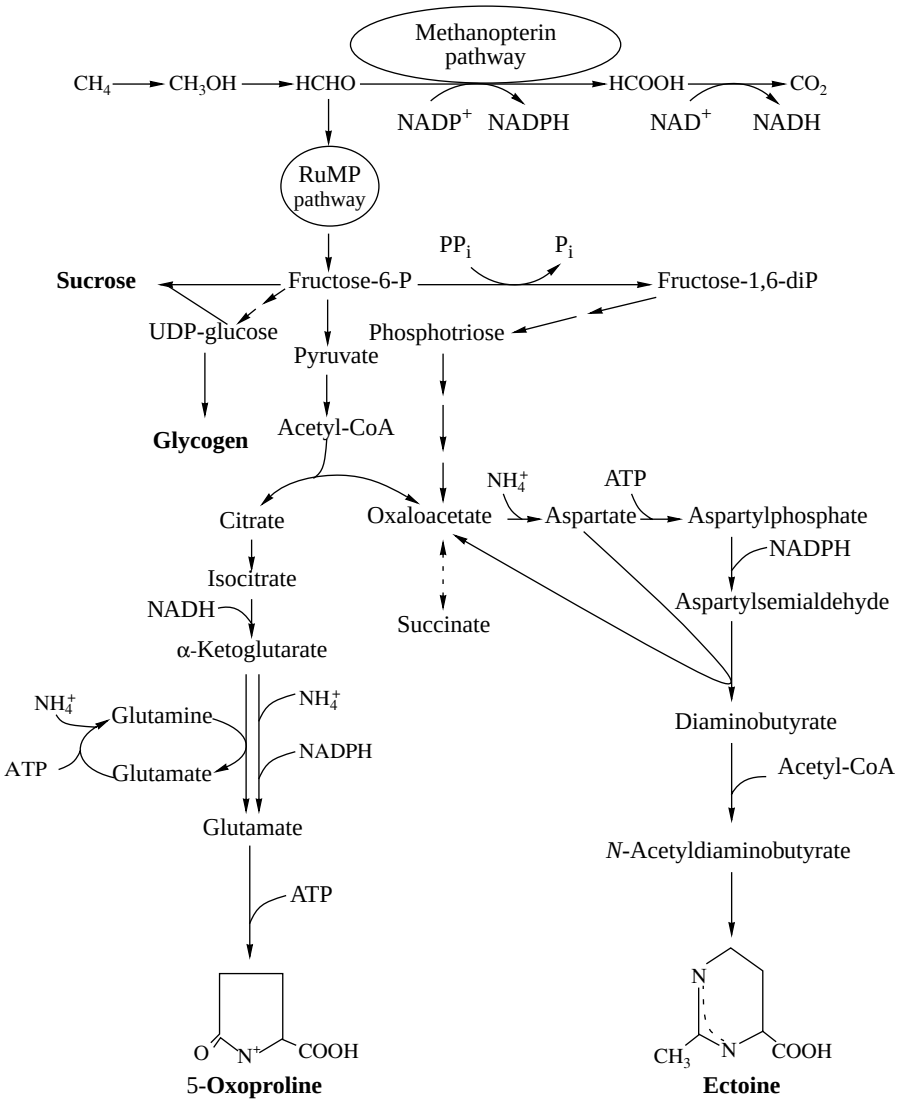

Fig. 2. Pathways of osmoprotectant synthesis in *M. alcaliphilum* 20Z.

phosphate synthase initiates the condensation of UDP-glucose (the glycogen precursor) and fructose-6-phosphate into sucrose-6-phosphate, which is then dephosphorylated with the formation of sucrose.

Another constitutive enzyme, glutamine synthetase, catalyzes the ATP-dependent cyclization of glutamic acid into pyroglutamate (5-oxoproline) in the absence of ammonium ions. The synthesis of glutamine and 5-oxoproline is controlled by the relative proportions of the intracellular concentrations of glutamate and of ammonium ions. In response to the increased salinity of the medium, halophilic and halotolerant microorganisms usually accumulate glutamate and its counterion, potassium. The accumulation of the osmoprotectant 5-oxoproline in obligate methanotrophs can be explained by the fact that the intracellular concentrations of the central metabolites, including glutamate, in these bacteria are low [49]. The conversion of glutamate into its cyclic and, therefore, more inert form 5-oxoproline can be considered as a mechanism for the discharge of glutamate in microbial cells grown at high salinities of the medium.

The biosynthesis of ectoine from aspartate includes several steps, which are essentially the same as in the halophilic bacteria *Ectothiorhodospira halochloris* and *Halomonas elongata* [50]. Aspartate is phosphorylated to aspartylphosphate, which is then converted into aspartylsemialdehyde, diaminobutyrate, and *N*-acetyldiaminobutyrate. The enzymes catalyzing the first two steps (aspartate kinase and aspartylsemialdehyde dehydrogenase) are also involved in the biosynthesis of amino acids of the aspartate family. All enzymes of this pathway in *M. alcaliphilum* 20Z cells grown in the presence of 1 M NaCl are very active. Methanotrophs are distinguished by the involvement of aspartate but

not glutamate in the transamination of aspartylsemialdehyde into diaminobutyrate (Fig. 2).

In the final analysis, the synthesis of 5-oxoproline and ectoine depends on the intracellular level of NADPH, as well as of pyruvate and acetyl-CoA, which come from the Entner–Doudoroff pathway. The major source of NADPH in aerobic methanotrophic bacteria is presumably the recently discovered methanopterin pathway [51], since the activity of NADP-dependent isocitrate dehydrogenase in these bacteria is low and cannot provide for the necessary level of NADPH. Glycolysis in obligate methanotrophs is blocked at the level of pyruvate kinase, causing a deficiency of glycolytic acetyl-CoA. This suggests that the main source of acetyl-CoA in these bacteria is also the methanopterin pathway [52]. The organization and regulation of the biosynthesis of osmoprotectants (ectoine, 5-oxoproline, and sucrose) in haloalkaliphilic methanotrophs can be better understood in the course of further physiological, biochemical, and molecular biological investigations.

The bioenergetic aspects of osmoadaptation. An important aspect of microbial adaptation to osmotic stress is the expenditure of energy for the maintenance of ionic homeostasis and osmoprotectant synthesis. The theoretical consideration that accounts for the metabolic specificity of methanotrophic bacteria showed that the synthesis of ectoine is more energy efficient than that of the other osmoprotectants [53]. At low salinities of the medium (such as 0.75% NaCl), or under the conditions of excess methane, or in the presence of methanol as the growth substrate, haloalkaliphilic and haloalkalitolerant methanotrophs accumulate sucrose and/or 5-oxoproline [48]. Ectoine is synthesized only at high salinities of the medium, when the maintenance of ionic gradients requires much energy and, therefore, the energy status of cells is low.

There is preliminary experimental evidence that methanotrophs possess specific bioenergetic mechanisms of osmoadaptation, such as the energy-dependent release of Na^+ ions from cells. The gradient of sodium ions (ΔpNa^+) can be used by the cells to provide energy for flagellum rotation and, hence, cell motility [28, 37]. Inhibition analysis showed that the gradient ΔpNa^+ in *M. alcaliphilus* 20Z cells is maintained both by the functioning of the electron-transport chain and by the hydrolysis of ATP. The fact that the motility of *M. alcaliphilum* 20Z cells is inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide suggests that Na^+-dependent NADH dehydrogenase may be involved in the extrusion of sodium ions from these cells and, hence, in the creation of the primary Na^+ gradient. Thus, the osmoadaptation of halophilic and halotolerant methanotrophs is associated with considerable structural and functional alterations, such as the modification of the chemical composition of the outer and inner membranes of these bacteria, the accumulation of organic (ectoine, 5-oxoproline, and sucrose) and inorganic (potassium ions) osmoprotectants, and the use of sodium bioenergetics. These mechanisms of osmoregulation and bioenergetics in (halo)alkaliphilic methanotrophs require closer investigations.

THE ECOPHYSIOLOGY AND BIOTECHNOLOGICAL POTENTIAL OF HALOALKALIPHILIC METHANOTROPHS

The ecophysiological role of methanotrophs. The experimental data that we obtained show that methanotrophs can live in bodies of salt and alkaline water and that (halo)alkaliphilic methanotrophs provide for the operation of the methane cycle in such extreme habitats. The methanotrophic bacteria inhabiting these ecosystems supply the products of methane oxidation (methanol, formaldehyde, and formate) for other members of the microbial communities, such as photo-, chemo-, hetero-, and methylotrophic bacteria [54].

The microorganisms of salt and soda lakes are known to perform specific stages of the nitrogen cycle, including nitrogen fixation, denitrification, and ammonification [14, 55, 56]. Nitrification also occurs in these ecosystems [57, 58], although the microorganisms that are responsible for the first step of this process are as yet unknown. It should be noted that the investigation of nitrification in alkaline ecosystems is of great interest, since ammonia (the main reduced form of nitrogen at pH 9.5 or higher) easily volatilizes, causing a deficiency of bound nitrogen.

The involvement of methanotrophs in the process of nitrification in soda lakes was first shown with reference to *M. kenii* AMO1. This bacterium, which was isolated from an African soda lake, efficiently oxidizes ammonium ions to nitrite at pH 10.0–10.5 [38]. Our investigations showed the occurrence of active processes of methane oxidation and formation of nitrite from ammonium ions in some soda lakes of Southern Transbaikal [29]. The methanotrophic bacterium *M. buryatense* 5B isolated from Lake Gorbunka was found to be able to oxidize ammonium ions to nitrite at pH values from 7 to 11, attaining optimal ability at pH 9.5–10.0. The real scale of nitrification in soda lakes is to be evaluated, but the involvement of methanotrophic bacteria in this process is beyond any doubt. Of interest is the ability of *M. kenii* AMO1 to oxidize carbon disulfide and the ability of some strains of the moderately halophilic marine methanotroph *Methylomicrobium* sp. N1 to oxidize trichloroethylene and dimethylsulfide. The strains of this bacterium that contain either soluble or particulate MMO oxidize dimethylsulfide into dimethylsulfoxide with equal intensities [38, 59]. It is obvious that methanotrophs, together with photo- and chemotrophs, are responsible for the maintenance of the organic matter pool in athalassic soda and salt lakes. The investigation of these ancient bodies of water may provide insight into the phylogeny and evolution of methanotrophic bacteria.

Biotechnological potential. In high-salinity environments, methanotrophs accumulate osmoprotectants, among which ectoine is of particular practical interest due to its high water-binding capacity and ability to exert a stabilizing effect on biomolecules (such as proteins and DNA) and whole cells [60, 61]. The investigation of the practical applications of ectoine is impeded by its high cost due to the complexity of its chemical synthesis and the absence of appropriate microbial producers. Haloalkaliphilic methanotrophs, which accumulate ectoine in large amounts, appear to be promising bacteria for developing new biotechnological processes of ectoine production.

The ability of haloalkaliphilic methanotrophs to form surface cell structures (S-layers) is also of great biotechnological and medical importance, since S-layers are considered to be potential nanotechnological elements for creating ultramembranes with very narrow molecular weight cut-off limits. The regular and close arrangement of functional groups in S-layers makes it possible to use them as matrices for the immobilization of bioactive macromolecules (enzymes and antibodies) in the production of biosensors. Furthermore, the S-layers can be used as affine chromatographic sorbents [40, 62].

The ability of some haloalkaliphilic methanotrophs to synthesize exopolysaccharides within wide ranges of pH, salinity, and methanol concentration can be used for the efficient production of exopolysaccharides from low-cost materials. The stable enzymes of haloalkaliphilic methanotrophs (for instance, methane monooxygenase and methanol dehydrogenase), which catalyze nonspecific oxidation reactions within wide ranges of pH and ionic strength, can also be used for the production of propylene oxide and other valuable chemical products.

Furthermore, the high ability of haloalkaliphilic methanotrophs to adapt to varying environmental conditions may be useful in various bioremediation processes, such as the removal of trichloroethylene and other toxic pollutants from saline and alkaline ecosystems. Further investigations of the metabolic potential of haloalkaliphilic methanotrophs may lead to other promising applications of these bacteria.

CONCLUSION

Considerable progress in the study of haloalkaliphilic methanotrophs offers great scope for the complex research of the methane cycle. The top-priority questions concern the following: (1) the structure of methanotrophic communities, particularly the occurrence and role of type II methanotrophs and those containing soluble MMO in saline and alkaline ecosystems; (2) the possibility of the existence and the life strategy of extremely halophilic methanotrophs capable of growing at salinities close to the saturation concentration of NaCl; (3) the kinetic properties of MMO in methanotrophic bacteria adapted to low concentrations of methane and oxygen; (4) the intensity of methane oxidation in extremely alkaline biotopes; (5) the nature of primary signal molecules initiating a sequence of structural and biochemical events in response to stress; and (6) the bioenergetic mechanisms of ionic homeostasis and the structure and diversity of genes controlling these mechanisms in methanotrophic bacteria and other microorganisms. These questions can be answered only by using complex approaches, such as the combination of molecular ecological methods with the direct measurement of gas flows in nature and the isolation of new methanotrophs with unusual properties.

ACKNOWLEDGMENTS

We are grateful to G.A. Zavarzin (Institute of Microbiology, Russian Academy of Sciences, Moscow), G. Gottschalk (Institute of Microbiology and Genetics, Gottingen, Germany), and all who contributed to this work.

This work was supported by grants nos. 98-04-48144 and 01-04-4851 from the Russian Foundation for Basic Research.

REFERENCES

1. Gal'chenko, V.F., Andreev, L.V., and Trotsenko, Yu.A., *Taksonomiya i identifikatsiya obligatnykh metanotrofnykh bakterii* (Taxonomy and Identification of Obligately Methanotrophic Bacteria), Pushchino: Nauchn. Tsentr Biol. Issled. Akad. Nauk SSSR, 1986.
2. Malashenko, Yu.P., Romanovskaya, V.A., and Trotsenko, Yu.A., *Metanokislyayushchie mikroorganizmy* (Methane-oxidizing Microorganisms), Moscow: Nauka, 1978.
3. Hanson, R.S. and Hanson, T.E., Methanotrophic Bacteria, *Microbiol. Rev.*, 1996, vol. 60, no. 2, pp. 439–471.
4. Sieburth, J.M., Jonson, P.W., Eberhardt, M.A., Sieracki, M.E., Lidstrom, M.E., and Laux, D., The First Methane-oxidizing Bacterium from the Upper Mixing Layer of the Deep Ocean: *Methylomonas pelagica* sp. nov, *Curr. Microbiol.*, 1987, vol. 14, pp. 285–293.
5. Hutton, W.E. and Zobell, C.E., The Occurrence and Characteristics of Methane-oxidizing Bacteria in Marine Sediments, *J. Bacteriol.*, 1949, vol. 58, no. 4, pp. 463–474.
6. Lidstrom, M.E., Isolation and Characterization of Marine Methanotrophs, *Antonie van Leeuwenhoek*, 1988, vol. 54, no. 3, pp. 189–199.
7. Gal'chenko, V.F., The Bacterial Methane Cycle in Marine Ecosystems, *Priroda*, 1995, no. 6, pp. 35–48.
8. Galchenko, V.F., *Ecology of Methanotrophic Bacteria in Aquatic Ecosystems*, Turpaev, T.M., Ed., New York: Harwood, 1995.
9. Malashenko, Yu.R., Khaier, Yu., Berger, U., Romanovskaya, V.A., and Muchnik, F.V., *Biologiya metanobrazuyushchikh i metanokislyayushchikh mikroorganizmov* (The Biology of Methanogenic and Meth-

ane-oxidizing Microorganisms), Kiev: Naukova Dumka, 1993.

10. Malashenko, Yu.R., Khaier, Yu., Romanovskaya, V.A., Berger, U., Budkova, E.N., and Shatokhina, E.S., Formation and Oxidation of Methane in Hypersaline Lakes, *Mikrobiol. Zh.*, 1995, vol. 57, no. 3, pp. 24–29.
11. Grant, W.D. and Jones, B.E., Alkaline Environments, *Encyclopedia of Microbiology*, Lederberg, J., Ed., New York: Academic, vol. 1, pp. 126–133.
12. Issatchenko, B.L., Biogenic Processes in the Chlorine, Sulfate, and Alkaline Lakes of the Kulunda Steppe, *Izbrannye trudy* (Selected Works), Moscow: Akad. Nauk SSSR, 1951, pp. 143–162.
13. Zhilina, T.N. and Zavarzin, G.A., Alkaliphilic Anaerobic Community at pH 10, *Curr. Microbiol.*, 1994, vol. 29, no. 2, pp. 109–112.
14. Zavarzin, G.A., Zhilina, T.N., and Pikuta, E.V., Secondary Anaerobes in the Haloalkaliphilic Communities of Tuva Lakes, *Mikrobiologiya*, 1996, vol. 65, no. 4, pp. 546–553.
15. Sorokin, D.Yu., Lysenko, A.M., and Mityushina, L.L., Isolation and Characterization of Alkaliphilic Chemoorganotrophic Bacteria Capable of Oxidizing Reduced Inorganic Sulfur Compounds to Tetrathionate, *Mikrobiologiya*, 1996, vol. 65, pp. 370–383.
16. Ventoza, A., Nieto, J.J., and Oren, A., Biology of Moderately Halophilic Aerobic Bacteria, *Microbiol. Mol. Biol. Rev.*, 1998, vol. 62, no. 2, pp. 504–544.
17. Yumoto, I., Yamasaki, K., Sawabe, T., Nakano, K., Kawasaki, K., Ezura, Y., and Shinano, H., *Bacillus horti* sp. nov., a New Gram-Negative Alkaliphilic Bacillus, *Int. J. Syst. Bacteriol.*, 1998, vol. 48, pp. 565–571.
18. Joye, S.B., Connell, T.L., Miller, L.G., Oremland, R.S., and Jellison, R.S., Oxidation of Ammonia and Methane in an Alkaline, Saline Lake, *Limnol. Oceanogr.*, 1999, vol. 44, no. 1, pp. 178–188.
19. Zavarzin, G.A., Epicontinental Soda Lakes as Possible Relic Biotopes Where Terrestrial Biota Had Evolved, *Mikrobiologiya*, 1993, vol. 62, no. 5, pp. 789–800.
20. Zavarzin, G.A., Zhilina, T., and Kevbrin, V.V., Alkaliphilic Microbial Communities and Their Functional Diversity, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 579–599.
21. Tindall, B.J., Prokaryotic Life in the Alkaline Saline Athalassic Environment, *Halophilic Bacteria*, Rodrigez-Valera, F., Ed., Boca Raton: CRC, 1988, pp. 31–67.
22. Jones, B.E., Grant, W.D., Collins, N.C., and Mwatha, W.E., Alkaliphiles: Diversity and Identification, *Bacterial Diversity and Systematics*, Priest, F.G. *et al.*, Eds., New York: Plenum, 1994, pp. 195–228.
23. Jones, B.E., Grant, W.D., Duckworth, A.W., and Owenson, G.G., Microbial Diversity of Soda Lakes, *Extremophiles*, 1998, vol. 2, pp. 191–200.
24. Gorlenko, V.M., Namsaraev, B.B., Kulyrova, A.V., Zavarzin, D.G., and Zhilina, T.N., Activity of Sulfate-reducing Bacteria in the Bottom Sediments of Soda Lakes in the Southeastern Transbaikal Region, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 664–670.
25. Slobodkin, F.I. and Zavarzin, G.A., Formation of Methane in the Halophilic Cyanobacterial Mats of Syvash, *Mikrobiologiya*, 1992, vol. 61, no. 2, pp. 294–299.
26. Conrad, R., Frenzel, P., and Cohen, Y., Methane Emission from Hypersaline Microbial Mats: Lack of Aerobic Methane Oxidation Activity, *FEMS Microbiology*, 1995, vol. 16, pp. 297–306.
27. Sokolov, A.P. and Trotsenko, Y.A., Methane Consumption in (Hyper)saline Habitats of the Crimea (Ukraine), *FEMS Microbiol. Ecol.*, 1995, vol. 18, pp. 299–304.
28. Khmelenina, V.N., Starostina, N.G., Tsvetkova, M.G., Sokolov, A.P., Suzina, N.E., and Trotsenko, Yu.A., Methanotrophic Bacteria in the Saline Lakes of Ukraine and Tuva, *Mikrobiologiya*, 1996, vol. 65, no. 5, pp. 696–703.
29. Khmelenina, V.N., Eshinimaev, B.Ts., Kalyuzhnaya, M.G., and Trotsenko, Yu.A., The Potential Activity of Methane and Ammonium Oxidation by the Methanotrophic Communities of Soda Lakes in the Southern Transbaikal Region, *Mikrobiologiya*, vol. 69, no. 4, pp. 553–558.
30. Namsaraev, B.B., Zhilina, T.N., Kulyrova, A.V., and Gorlenko, V.M., Bacterial Production of Methane in the Soda Lakes of the Southeastern Transbaikal Region, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 671–676.
31. Murrell, J.C., McDonald, I.R., and Bourne, D.G., Molecular Methods for the Study of Methanotroph Ecology, *FEMS Microbiol. Ecol.*, 1998, vol. 27, pp. 103–114.
32. Holmes, A.J., Owens, N.J., and Murrell, J.C., Detection of Novel Marine Methanotrophs Using Phylogenetic and Functional Gene Probes after Methane Enrichment, *Microbiology*, 1995, vol. 141, no. 8, pp. 1947–1955.
33. Khmelenina, V.N., Kalyuzhnaya, M.G., Starostina, N.G., Suzina, N.E., and Trotsenko, Y.A., Isolation and Characterization of Halotolerant Alkaliphilic Methanotrophic Bacteria from Tuva Soda Lakes, *Curr. Microbiol.*, 1997, vol. 35, no. 5, pp. 257–261.
34. Kalyuzhnaya, M.G., Khmelenina, V.N., Starostina, N.G., Baranova, S.V., Suzina, N.E., and Trotsenko, Yu.A., A New Moderately Halophilic Methanotroph of the Genus *Methylobacter*, *Mikrobiologiya*, 1998, vol. 67, no. 4, pp. 532–539.
35. Kalyuzhnaya, M.G., Khmelenina, V.N., Suzina, N.E., Lysenko, A.M., and Trotsenko, Yu.A., New Methanotrophic Bacteria Isolated from the Soda Lakes of Southern Transbaikal, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 689–697.
36. Kalyuzhnaya, M.G., Khmelenina, V.N., Eshinimaev, B.Ts., Suzina, N.E., Nikitin, D., Solonin, A., Lin, J.L., McDonald, I.R., Murrell, J.C., and Trotsenko, Y.A., Taxonomic Characterization of New Alkaliphilic and Alkalitolerant Methanotrophs from Soda Lakes of the Southeastern Transbaikal Region and Description of *Methylomicrobium buryatense* sp. nov., *Syst. Appl. Microbiol.*, 2001, vol. 24, pp. 166–176.
37. Khmelenina, V.N., Kalyuzhnaya, M.G., and Trotsenko, Yu.A., Physiological and Biochemical Characteristics of the Haloalkalitolerant Methanotroph *Methylobacter alcaliphilus*, *Mikrobiologiya*, 1997, vol. 66, no. 4, pp. 447–453.
38. Sorokin, D.Y., Jones, B.E., and Kuenen, J.G., A Novel Obligately Methylotrophic, Methane-oxidizing *Methylomicrobium* Species from a Highly Alkaline Environment, *Extremophiles*, 2000, vol. 4, no. 1, pp. 145–155.
39. Khmelenina, V.N., Kalyuzhnaya, M.G., Sakharovsky, V.G., Trotsenko, Y.A., and Gottschalk, G., Osmoadaptation in

Halophilic and Alkaliphilic Methanotrophs, *Arch. Microbiol.*, 1999, vol. 172, no. 5, pp. 321–329.

40. Severina, L.O., Bacterial S-layers, *Mikrobiologiya*, 1995, vol. 64, no. 6, pp. 725–733.
41. Sleytr, U.B. and Beveridge, J., Bacterial S-layers, *Trends Microbiol.*, 1999, vol. 7, no. 6, pp. 253–260.
42. Tyurin, V.S., Gorskaya, L.A., Kaftanova, A.S., Loginova, T.M., and Mikhailov, A.M., Some Particular Characteristics of the Minute Structure of *Methylococcus capsulatus* Cells under Different Cultivation Conditions, *Mikrobiologiya*, 1985, vol. 54, no. 6, pp. 770–775.
43. Russell, N.J., Adaptive Modifications in Membranes of Halotolerant and Halophilic Microorganisms, *J. Bioenerg. Biomembr.*, 1989, vol. 54, no. 1, pp. 93–113.
44. Thiemann, B. and Imhoff, I.F., The Effect of Salt on the Lipid Composition of *Ectothiorhodospira*, *Arch. Microbiol.*, 1991, vol. 156, no. 5, pp. 376–384.
45. Galinski, E.A. and Trüper, H.G., Microbial Behavior in Salt-stressed Ecosystems, *FEMS Microbiol. Rev.*, 1994, vol. 15, pp. 95–108.
46. Galinski, E.A., Osmoadaptation in Bacteria, *Adv. Microb. Physiol.*, 1995, vol. 37, pp. 273–328.
47. Costa, M.S., Santos, H., and Galinski, E.A., Solutes in Bacteria and Archaea, *Adv. Biochem. Eng. Biotechnol.*, 1998, vol. 61, pp. 118–153.
48. Khmelenina, V.N., Sakharovskii, V.G., Reshetnikov, A.S., and Trotsenko, Yu.A., Synthesis of Organic Osmoprotectants in Halophilic and Alkaliphilic Methanotrophs, *Mikrobiologiya*, vol. 69, no. 4, pp. 465–470.
49. Shishkina, V.N. and Trotsenko, Y.A., Multiple Metabolic Lesions in Obligate Methanotrophic Bacteria, *FEMS Microbiol. Lett.*, 1982, vol. 13, pp. 237–242.
50. Peters, P., Galinski, E.A., and Trüper, H.G., The Biosynthesis of Ectoine, *FEMS Microbiol. Lett.*, 1990, vol. 71, no. 1-2, pp. 157–162.
51. Vorholt, J.A., Chistoserdova, L., Stolyar, S.M., Thauer, R.K., and Lidstrom, M.E., Distribution of Tetrahydromethanopterin-Dependent Enzymes in Methylotrophic Bacteria and Phylogeny of Methenyl Tetrahydromethanopterin Cyclohydrolases, *J. Bacteriol.*, 1999, vol. 181, no. 18, pp. 5750–5757.
52. Schauder, R., Preuß, A., Jetten, M., and Fuchs, G., Oxidative and Reductive Acetyl CoA/Carbon Monoxide Dehydrogenase Pathway in *Desulfobacterium autotrophicum*: II. Demonstration of the Enzymes of the Pathway and Comparison of CO Dehydrogenases, *Arch. Microbiol.*, 1989, vol. 151, no. 1, pp. 84–89.
53. Oren, A., Bioenergetic Aspects of Halophilism, *Microbiol. Mol. Biol. Rev.*, 1999, vol. 63, no. 2, pp. 334–348.
54. Doronina, N.V., Darmaeva, Ts.D., and Trotsenko, Yu.A., New Aerobic Methylotrophic Isolates from the Soda Lakes of Southern Transbaikal, *Mikrobiologiya*, 2001, vol. 70, no. 3, pp. 398–404.
55. Kevbrin, V.V., Zhilina, T.N., Rainey, F.A., and Zavarzin, G.A., *Tindallia magadii* gen. nov., sp. nov., an Alkaliphilic Anaerobic Ammonifier from Soda Lake Deposits, *Curr. Microbiol.*, 1998, vol. 37, pp. 94–100.
56. Zhilina, T.N., Detkova, E.N., Rainey, F.A., Osipov, G.A., Lysenko, A.M., Kostrikina, N.A., and Zavarzin, G.A., *Natronoincola histidinovorans* gen. nov., sp. nov., a New Alkaliphilic Acetogenic Anaerobe, *Curr. Microbiol.*, 1998, vol. 37, pp. 177–185.
57. Ward, B.B., Martino, P.P., Diarz, M.C., and Joe, S.B., Analysis of Ammonia-oxidizing Bacteria from Hypersaline Mono Lake, California, on the Basis of 16S RNA Sequences, *Appl. Environ. Microbiol.*, 2000, vol. 66, no. 7, pp. 2873–2884.
58. Joye, S.B., Connell, T.L., Miller, L.G., Oremland, R.S., and Jellison, R.S., Oxidation of Ammonia and Methane in an Alkaline, Saline Lake, *Limnol. Oceanogr.*, 1999, vol. 44, no. 1, pp. 178–188.
59. Fuse, H., Ohta, M., Takimura, O., Murakami, K., Inoue, H., Yamaoka, Y., Oclarit, J.M., and Omori, T., Oxidation of Trichloroethylene and Dimethyl Sulfide by a Marine *Methylomicrobium* Strain Containing Soluble Methane Monooxygenase, *Biosci. Biotechnol. Biochem.*, 1998, vol. 62, pp. 1925–1931.
60. Lippert, K. and Galinski, E.A., Enzyme Stabilization by Ectoine-Type Compatible Solutes: Protection against Heating, Freezing, and Drying, *Appl. Microbiol. Biotechnol.*, 1992, vol. 37, pp. 61–65.
61. Louis, P., Trüper, H.G., and Galinski, E.A., Survival of *Escherichia coli* during Drying and Storage in the Presence of Compatible Solutes, *Appl. Microbiol. Biotechnol.*, 1994, vol. 41, pp. 684–688.
62. Sleytr, U.B., Basic and Applied S-Layer Research: An Overview, *FEMS Microbiol. Rev.*, 1997, vol. 20, no. 1/2, pp. 5–12.
63. Kevbrin, V.V., Zhilina, T.N., and Zavarzin, G.A., The Degradation of Cellulose by an Anaerobic Alkaliphilic Microbial Community, *Mikrobiologiya*, 1999, vol. 68, no. 5, pp. 686–695.