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EXPERIMENTAL ARTICLES

Structural and Functional Features of Methanotrophs from Hypersaline and Alkaline Lakes

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Abstract—Ten strains of aerobic methanotrophic bacteria represented by halophilic neutrophiles or halotolerant alkaliphiles were isolated from saline and alkaline lakes of southeast Siberia, Mongolia, Africa, and North America. Based on analysis of the nucleotide sequences of 16S rRNA gene and the *pmoA* gene encoding particulate methane monooxygenase, the isolates were classified as *Methylomicrobium alcaliphilum, Methylomicrobium buryatense*, and *Methylobacter marinus*. All strains of the genus *Methylomicrobium* were shown to synthesize glycoprotein S-layers located on the cell surface with hexagonal symmetry (*p*6) as a monolayer of cup-shaped structures or fine "inverted" conical structures and as plates consisting of protein subunits with inclined (*p*2) symmetry. During adaptation to the high salinity of the medium, isolated methanotrophs synthesize osmoprotectants: ectoine, sucrose, and glutamate. The *ectC* gene encoding ectoine synthase (EctC) was identified in six methanotrophic strains. Phylogenetic analysis of translated amino acid sequence of the *ectC* gene fragment suggests lateral transfer of the genes of ectoine synthesis as the most probable way for methanotrophs to acquire resistance to high external salinity.

Key words: halophilic and alkaliphilic methanotrophs, taxonomy, phylogeny, S-layers, osmoadaptation, ectoine, *Methylomicrobium, Methylobacter*.

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Aerobic bacteria utilizing methane as a carbon and energy source (methanotrophs) have been recently found in hypersaline and highly alkaline ecosystems [1–7]. Methanotrophs adapted to high salinity and/or pH values are geno- and phenotypically different from the inhabitants of freshwater environments with neutral pH values and have been described as new species of the genera *Methylomicrobium* and *Methylobacter* [4] and the recently proposed genus *Methylohalobius* [6].

We have shown that formation on their cell surface of glycoprotein S-layers is a characteristic feature of the ultrastructure of alkaliphilic and halophilic methanotrophs of the genus *Methylomicrobium*; the functions of these structures are still unclear [7, 8]. In addition, these bacteria synthesize organic osmolytes for equalization of osmotic pressure between the cytoplasm and the medium, the major osmoprotectant being the cyclic amino acid ectoine [8, 9]. This multifunctional bioprotectant widely distributed in bacteria is actively used in research practice, medicine, and cosmetics [10]. The pathway of ectoine synthesis is a branch in the biochemical sequence of synthesis of the aspartate family amino acids, represented by three specific enzymes: diaminobutyrate acetyltransferase (EctA), diaminobutyrate aminotransferase (EctB), and ectoine synthase (EctC). In *Mm. alcaliphilum* 20Z, the genes encoding the enzymes of ectoine synthesis form an *ectABCask* operon carrying an additional gene of aspartate kinase [11]. It would be logical to assume that further study of the distribution of methanotrophs in geographically distant saline and soda lakes with different hydrochemistry (including taxonomic diversity and physiological and cytological characteristics) will shed more light onto the adaptation mechanisms of these bacteria to various extreme factors and extend their biotechnological potential.

The goal of this work was the taxonomic and structural and functional characterization of methanotrophs isolated from saline and soda lakes of different geographical regions.

MATERIALS AND METHODS

Objects of research. The silt samples from soda and saline lakes were kindly provided by D.Yu. Sorokin and V.M. Gorlenko (Institute of Microbiology, Russian Academy of Sciences). The pH and general salinity values of these lakes are given in Table 1. The halo(alkali)philic methanotrophs isolated from these environments were the objects of the research. The

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Table 1. Key features of the sampling sites and the properties of new isolates

Lake	Sampling site characteristics		Strains	ICM type (C_1 assimilation pathway)	DNA G+C, mol %	Growth conditions		
	pH	Total salinity, g/l				Temperature, °C	pH range (optimum)[1]	NaCl, % (optimum)
Mongolia								
Khotontyn	9.7–10.3	360	FM3	I (RuMP)	50.5	8–30	6.8–10.5 (8.6)	0–6 (1.3)
48°N, 115°E								
Egypt								
Fazda	9.7	250	E3	I (RuMP)	50	8–37	6.8–10.5 (9.0)	0–9 (0.8)
30°N, 30°E								
United States								
MonoLake	9.5	120	ML1	I (RuMP)	50.5	8–30	6.8–10.3 (9.0)	0–7 (1.0)
38°N, 119°W								
Kulunda steppe, Altai krai, Russia								
Uinak	9.3–9.6	20–46	1S	I (RuMP)	nd	26	6.8–10.3 (9.5)	0–8 (2)
53°N, 78°E								
Tanatar	9.9	30–82	2S	I (RuMP)	nd	26	6.8–10.3 (9.5)	0–8 (2)
51°N,79°E								
Petukhovo	9.9	54	3S	I (RuMP)	nd	26	6.8–10.3 (9.5)	0–9 (2)
52°N, 79°E								
Iodnoe	9.1–9.3	108–150	4S	I (RuMP)	nd	26	6.5–10.5 (9.5)	0–8 (2)
Lechebnoe	9.1–9.3	108–150	4S1	I (RuMP)	nd	26	6.8–10.3 (9.5)	0–8 (2)
Nikolaevskoe	9.3–9.5	100–118	5S	I (RuMP)	nd	26	6.5–10 (9.5)	0–8 (2)
Kulundinskoe	7.8–8.2	226–283	3C[2]	I	nd	29	7.5	Up to 8 (6)
53°N, 79°E								
Burlinskoe	7.45	360	7C	I (RuMP)	51.5	29	6.2–9.0 (7.5)	1–4 (2)
53°N, 78°E								

[1] The optimal pH and salinity values are given in parentheses.

[2] Enrichment culture was not divided into components; RuMP, ribulose monophosphate cycle; nd, not determined.

type strains of haloalkaliphilic methanotrophs *Mm. alcaliphilum* 20Z^T and *Mm. buryatense* 5B^T were used for comparative studies.

Isolation of enrichment and pure cultures of methanotrophic bacteria. Enrichment cultures of methanotrophs were obtained by introducing 1 g of lake sediment samples into 30–50 ml of P mineral medium [12]. The pH value of the medium was adjusted in accordance with the pH values of the natural sources by adding phosphate (pH 6.5–8.0), Na–carbonate (pH 8–10.5), or NaOH–phosphate (pH 11) buffers to the final concentration of 50–100 mM. The media were supplemented with NaCl in a concentration corresponding to the lake salinity (0.2–20%). Incubation was performed in a methane/air (1 : 1) atmosphere for 1–4 weeks at 29°C on a rotary shaker (140 rpm). The time between successive passages was decreased as the share of methanotrophs in the enrichment culture increased. In some cases, the culture was enriched with methanotrophs by separating the latter from smaller heterotrophic satellites by filtration through sterile membrane filters (pore size 0.45–0.8 μm) or by centrifugation (3000 *g*, 3 min). After recentrifugation of the supernatant at 2000 *g* for 5 min, the cells were resuspended and washed with sterile medium on the filters which were then used as inocula. Pure cultures were isolated by plating on media with 1.5–2% Difco agar (United States). The purity of cultures was controlled by light and electron microscopy and by the absence of growth on organic media: glucose–potato agar [12] and peptone–yeast medium (0.2% peptone, 0.1% yeast autolysate, and 1.5% Difco agar).

Physiological and biochemical characterization of the isolates. The effect of salt concentration on the growth rates of methanotrophs was studied by growing isolates in P medium supplemented with 0–10%

NaCl, pH 9.5 or 7.2 for alkaliphilic and neutrophilic strains, respectively. The effect of pH was studied by cultivation of the cells under optimal salinity in the pH range of 6.5–11.0.

The presence of soluble methane monooxygenase (sMMO) was determined by naphthalene oxidation by the cells [13]. The colonies of methanotrophic bacteria were grown on P medium without copper. The plates were stored for 1 h in a desiccator, with naphthalene crystals placed onto its bottom. Tetrazotized *o*-dianisidine solution (5 mg/ml) was poured onto the agar surface. The colonies of sMMO-containing strains were stained purple. Enzyme activities were measured by the previously described methods in the extracts obtained from the exponentially grown cells harvested by centrifugation (6000 *g*, 15 min) and washed with 0.05 M Tris–HCl (pH 7.5) at the relevant NaCl concentration [1]. The methods used for analysis of fatty acids and detection of osmoprotectants have been described previously [8].

Electron microscopy. Cell fixation, ultrathin sectioning, freeze-fracturing, and analysis of the preparations in a JEOL JEM 100B electron microscope (Japan) were performed as described in [8].

Determination of DNA G+C content and PCR amplification. DNA was isolated and purified as described [14]. DNA G+C base content was determined by melting temperature in a Pye Unicam SP 1800 spectrophotometer (United Kingdom) [1]. PCR amplification of the fragments of the *pmoA* gene coding for particulate monooxygenase was performed at 56°C using the pair of primers A189f and mb661r [15, 16] in an Eppendorf thermocycler (Germany). Fragments of the *ectB–ectC* genes were amplified using degenerate primers 5'-ACCGG(T/C)-ACITT-(C/T)TT(C/T)AGITT(C/T)GA-3' (corresponding to the *ectB* gene) and 5'-GGIGG(A/G)T-T(A/G)AANAC(A/G)CA-3' (corresponding to the *ectC* gene) as described [11]. DNA sequencing was performed at the GENOME Interinstitute Center (Institute of Molecular Biology, Russian Academy of Sciences) in an ABI PRISM automatic sequencer (United States) using the ABI PRISM RRigDye™ Terminator v. 3.1 reagent kit. The nucleotide sequences were translated into amino acid sequences using the Vector NTI 9.1 software package. Translated amino acid sequences of the *pmoA* gene region (124 aa) and nucleotide sequences of the 16S rRNA gene (948 bp) were compared with the GenBank sequences using the NCBI BLAST software package (http//www.ncbi.nlm.nih.gov/Blast). The sequences were aligned using the BioEdit 7.0.1 software package. Phylogenetic trees were constructed using the Treecon W (1.3b) and MEGA 4 software packages.

RESULTS AND DISCUSSION

Physiological properties of the new isolates. The diversity of aerobic methanotrophs in geographically remote saline and soda lakes differing in pH values (7.45–10) and general mineralization (20–360 g/l) was estimated by an approach based on obtaining of the most adapted forms of methanotrophic bacteria. Bottom sediment samples were incubated in the methane/air (1 : 1) atmosphere in liquid mineral media with pH and NaCl concentration corresponding to the in situ values. As a result, 11 enrichment cultures of aerobic methanotrophic bacteria were isolated from the saline and soda lakes of the Kulunda steppe (Russia), Mongolia, Egypt, and North America. Ten pure methanotrophic cultures were then isolated using special enrichment techniques for methanotrophic cultures (differential centrifugation and filtration through membrane filters) (Table 1). As a rule, a substantial decrease of salt concentration in the medium was needed to obtain pure cultures of methanotrophs.

During growth on solid media for 4–7 days at 26°C, the cultures formed round, convex, cream-colored colonies, 2–4 mm in size. The cells were gram-negative rods, 1.2–2.7 × 0.7–1.2 μm. The new isolates were mesophiles growing in the range of 8–37°C, optimally at 30°C. Strains 1S, 2S, 3S, 4S, 4S1, 5S, ML1, FM3, and E3 isolated from soda lakes grew at pH 6.5–10.3 and salinity up to 8–9% NaCl, with the maximum rate at pH 9.0–9.5 and 0.5–2% NaCl (Table 1). It should be noted that, for growth in both alkaline and neutral media, these alkaliphiles required Na^+ ions, which were added to the medium as carbonates or sodium chloride in the concentration of 0.05 M (Fig. 1).

The strain 7C growing in the pH range of 6.5–9.5 at a salinity of 0.5–5% NaCl (Fig. 1) was isolated from Lake Burlinskoe with neutral pH values. The strain 7C was obligately dependent on sodium chloride and grew at a maximum rate at pH 7.5 and 2% NaCl. Thus, the isolated strains were either halotolerant facultative alkaliphiles or neutrophilic moderate halophiles (strain 7C). The isolates grew on methane or methanol as a carbon and energy source but were incapable of growth on polycarbon substrates. Nitrates, nitrites, ammonium salts, yeast extract, urea, casamino acids, glutamate, and glycine were used as nitrogen sources, with the exception of strain 3S, which did not grow on the medium with glycine.

The physiological properties of the new isolates only partially corresponded to the conditions of hypersaline and soda lakes, since in pure cultures they usually grew at a much lower salinity. For *Mm. alcaliphilum* FM3 and *Mb. marinus* 7C, the salinity values optimal for growth (1.3 and 2% NaCl) and the maximum permissible salt concentration (5–6% NaCl) were much lower than the water salinity in the lakes from which these cultures had been isolated (36% NaCl) (Table 1). This demonstrates a close

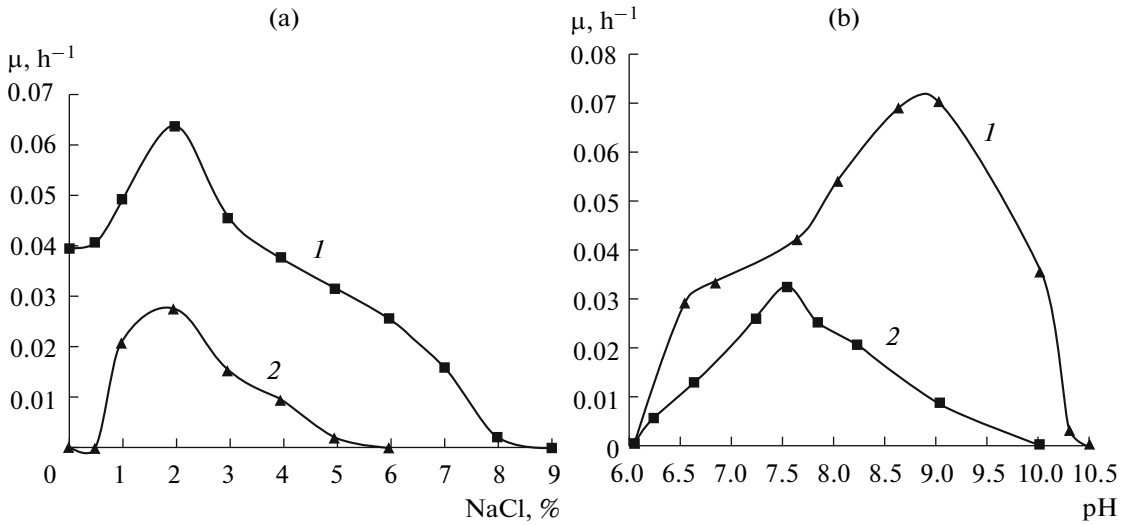


Fig. 1. Growth rates of strains 5S (*1*) and 7C (*2*) depending on salinity (a) and pH (b) of the growth medium.

interrelationship between the components of microbial communities in these ecosystems; it is likely the case that survival at a high salinity is favored by mutual exchange of osmolytes. It is notable that the addition of the supernatant of the culture liquid from the primary enrichments increased the salt tolerance of pure methanotrophic cultures.

Metabolic characteristics. The cells of methanotrophs did not oxidize naphthalene and, therefore, did not possess sMMO. Moreover, the sMMO-encoding gene was not found by PCR with the respective primers targeting the α subunit of the enzyme. Enzymological analysis of cell-free extracts revealed the presence of methanol, formaldehyde, and formate dehydrogenases showing activity with phenazine methosulfate (PMS) as an artificial electron acceptor. Formate dehydrogenase was active also with NAD^+ (Table 2). The high activities of the hexulose phosphate synthase indicated the operation of the ribulose monophosphate (RuMP) pathway of formaldehyde fixation in the isolates. The presence of the activities of 2-keto-3-deoxy-6-phosphogluconate aldolase and fructose-1,6-bisphosphate aldolase indicated the functioning of two pathways for phosphosugars cleavage: the Entner–Doudoroff pathway and the Embden–Meyerhof–Parnas pathway; in the latter case, the reaction of fructose-6-phosphate phosphorylation was catalyzed by PPi- but not ATP-dependent 6-phosphofructokinase. Moreover, the oxidative pentose phosphate pathway of phosphosugar splitting in these bacteria involved $NAD(P)^+$ dependent glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Activities of the enzymes of the serine pathway (hydroxypyruvate reductase and serine-glyoxylate aminotransferase) and the Calvin cycle (ribulose bisphosphate carboxylase) were not found. All strains lacked the α-ketoglutarate dehydrogenase activity; therefore, the incomplete Krebs cycle performed, mainly a biosynthetic function. Activities of the glyoxylate bypass enzymes (isocitrate lyase and malate synthase) were also absent. The isolates assimilated NH_4^+ via the glutamate cycle and by means of reductive amination of α-ketoglutarate and pyruvate with participation of glutamate synthase, glutamine synthetase, and glutamate and alanine dehydrogenases, respectively. Generally, the metabolic design of the isolates was analogous to that of other methanotrophs of morphotype I. It is obvious that the employment of the RuMP cycle of formaldehyde fixation, being the most energetically efficient of the known C_1 assimilation pathways, gives methanotrophs an advantage in osmoadaptation, in particular, maintenance of the ionic cell homeostasis [7].

Molecular identification of the isolates. The DNA G+C content of the isolates (50–51.5 mol %) was typical of the type I methanotrophs. Analysis based on the comparison of the *pmoA* gene fragment encoding one of the subunits of membrane MMO showed high similarity of the isolated alkaliphilic methanotrophs with *Mm. buryatense* (99.1% identity of translated amino acid sequences). The strain 4S showing maximum similarity with *Mm. alcaliphilum* (100% identity) (Fig. 2) was an exception. However, comparison of the 16S rRNA gene sequences classified of the isolates from soda lakes as *Mm. alcaliphilum* (99.4–99.7% similarity), except for the strain 3S, which was close to *Mm. buryatense* (Fig. 3). As a rule, the phylogeny of methanotrophs based on the 16S rDNA sequence completely corresponds to that of the *pmoA* gene [16]. However, our data showed that the species affiliation of methanotrophs could not be unambiguously established only by the amino acid sequence of the PmoA.

According to the genotypic properties, the neutrophilic halophilic strain 7C proved to be the closest rel-

Table 2. The enzyme activities in the new (halo)alkaliphilic methanotrophs (nmol min^{-1} mg^{-1} protein)

Enzyme	Cofactor	Strain									
		FM3	E3	ML1	1S	2S	3S	4S	4S1	5S	7C
Methanol dehydrogenase	PMS	342	96	50	179	227	129	201	68	270	80
Formaldehyde dehydrogenase	PMS	30	18	8	10	36	4	37	22	12	57
	NAD^+	12	10	0	0	21	24	58	3	4	20
Formate dehydrogenase	PMS	152	112	164	52	62	25	128	45	42	62
	NAD^+	12	11	8	14	14	26	129	25	5	25
Hexulose phosphate synthase		59	76	23	152	90	28	257	54	67	98
Glutamate dehydrogenase	NADH	19	19	18	4	6	10	2	2	nd	10
	NADPH	0	nd	nd	2	9	6	2	2	nd	10
Alanine dehydrogenase	NADH	nd	nd	nd	9	6	2	3	4	2	16
	NADPH	nd	nd	nd	5	5	8	3	3	7	5
Glutamate synthase	NADH	23	30	24	7	4	7	2	2	1	7
	NADPH	10	15	5	2	6	7	2	3	0	11
Glutamine synthetase	Mn^{2+}, ATP	21	19	34	278	106	43	84	73	62	4
6-Phosphogluconate dehydrogenase	$NADP^+$	nd	nd	nd	4	6	5	5	3	1	7
Glucose-6-phosphate dehydrogenase	NAD^+	0	0	0	3	1	0	3	0	2	36
	$NADP^+$	11	6	8	3	3	4	4	4	4	7
KDPG-aldolase	NADH	18	13	17	6	16	21	18	6	10	11
FBP-aldolase	NADH	132	167	106	39	10	8	32	3	18	17
PPi-phosphofructokinase	NADH	36	35	20	10	9	17	129	9	22	16

Note: Cell extracts of the strains under study did not exhibit the activities of hydroxypyruvate reductase, serine-glyoxylate aminotransferase, ribulose bisphosphate carboxylase, and α-ketoglutarate dehydrogenase. KDPG, 2-keto-3-deoxy-6-phosphogluconate; FBP, frucrose-1,6-bisphosphate; PPi, inorganic pyrophosphate; nd, not determined.

ative of *Methylobacter marinus* (99.3% identity of the *pmoA* gene sequence and 99.3% similarity of the 16S rRNA gene) [17]. In addition, the monoculture 3C growing in the neutral medium at 6% NaCl was isolated from silt of the hypersaline Lake Malinovoe in the Kulunda steppe. The PmoA sequence of this methanotroph showed the highest identity with *Methylohalobius crimeensis* (99.1%) [6].

Thus, the isolated methanotrophs belonged mainly to the species *Mm. alcaliphilum*, which indicates its wide distribution in soda lakes of different geographical regions. On the contrary, the halophilic methanotrophs close to *Mb. marinus* and *Methylohalobius crimeensis*, initially described as inhabitants of marine ecosystems, were isolated from hypersaline lakes of the Kulunda steppe with neutral pH values of the water [6, 17].

Fatty acid composition. Fatty acid profiles of the isolates affiliated, according to the 16S rDNA sequence, to the genera *Methylomicrobium* or *Methylobacter* were practically identical and close to those of the previously described alkaliphilic halotolerant methanotrophs [3]. Predominant fatty acids were monounsaturated hexadecanoic acids ($C_{16:1}\omega7C$), but the isomer $C_{16:1}\omega8C$, typical of neutrophilic nonhalophilic methanotrophs of the genus *Methylomicrobium*, was absent (Table 3). Consequently, at least in salt-dependent methanotrophs, fatty acid profiles should not be considered as a generic taxonomic marker. The composition and ratio of fatty acids in the strain 5S were practically unchanged when pH values of the cultivation medium varied (pH 9.0 or pH 7.2) (Table 3).

Ultrastructural properties of the cells. The cell wall structure of the isolated strains was typical for gram-negative bacteria (Fig. 2). The cytoplasm contained intracytoplasmic membranes (ICM) of morphotype I as stacks of vesicular discs and inclusions similar to the glycogen granules in *Mm. alcaliphilum* 20Z and *Mm. buryatense* 5B [8, 18].

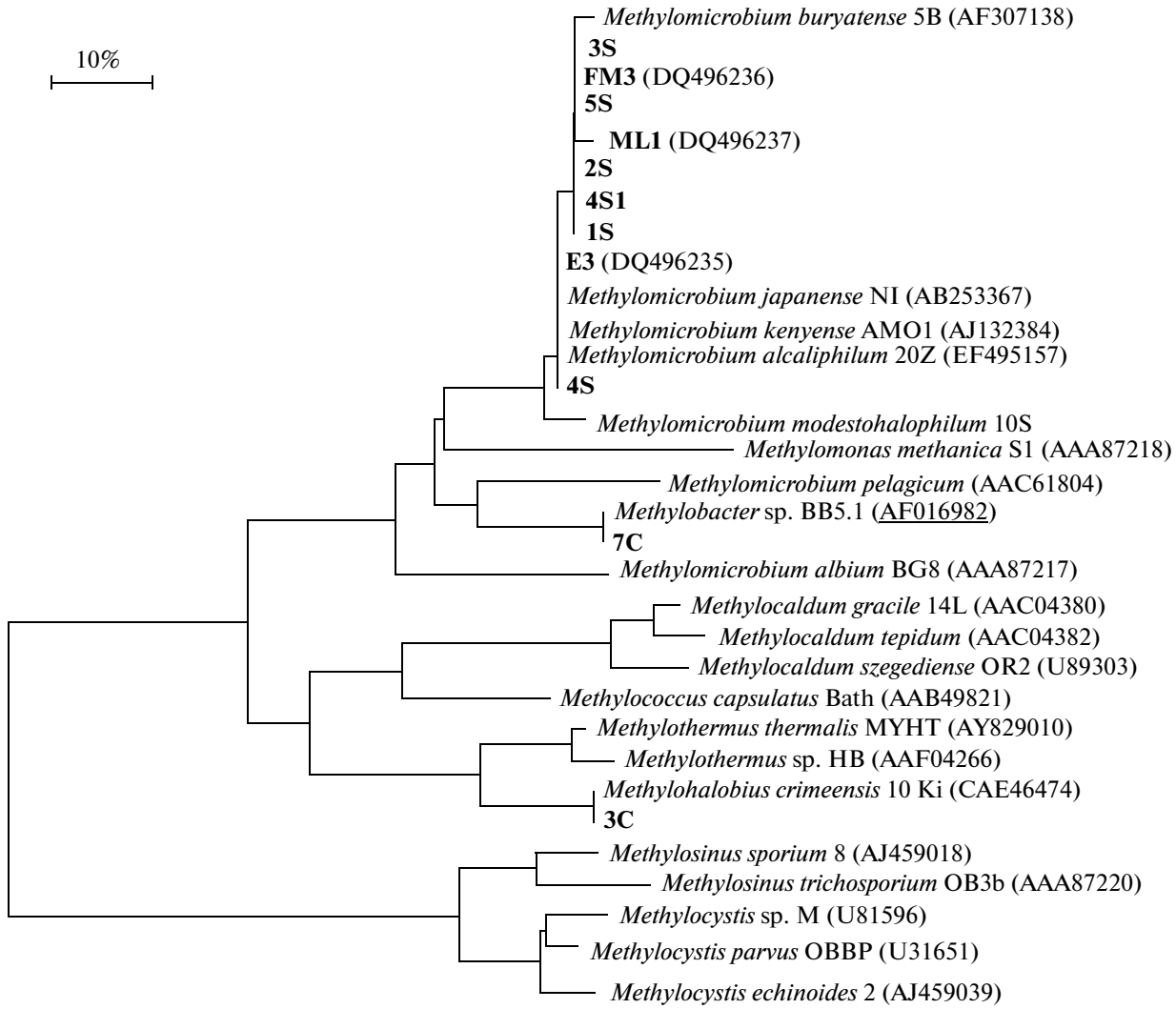


Fig. 2. Phylogenetic tree constructed on the basis of comparative analysis of translated amino acid sequences of the *pmoA* gene of the new isolates and other methanotrophic bacteria. The tree was constructed relative to the AmoA fragment of *Nitrosococcus oceanus*.

Regular S-layers were found on the cell surface of strains 1S and 2S. These layers were represented by cup-shaped structures 36 nm in height and 33 nm in diameter arranged with hexagonal (*p*6) symmetry and identical in morphology to the S-layers of *Mm. alcaliphilum* 20Z (Figs. 4a, 4d, 4g) [1, 8]. The packing density of cup-shaped structures was 900 U/μm^2. In strain 3S, cup-shaped structures (40 nm in height and 29 nm in diameter) had denser hexagonal packing (1225 U/μm^2) and were analogous to those of *Mm. buryatense* (Fig. 4j) [3]. On the contrary, leaf-shaped S-layers consisting of the protein subunits with linear (*p*2) symmetry were found on the cell surface of *Mm. alcaliphilum* ML1 (Figs. 4c, 4f, 4i).

Another morphotype of S-layers was found in *Mm. alcaliphilum* strains FM3, E3, 4S, 4S1, and 5S. Analysis of ultrathin sections and cryofractures of the 5S cells showed that these S-layers consisted of a monolayer of "inverted" conical structures 18 nm in height and 13 nm in diameter arranged in the *p*6 symmetry (Figs. 4b, 4e, 4h). Such S-layers, previously described as globular surface structures, are formed also by the alkaliphilic methanotroph *Mm. kenyense* AMO1 [2]. Although no S-layers were detected in the strains 3C and 7C, the strain 7C was shown to have membrane vesicles, the functions of which still have to be elucidated (Fig. 4k).

Thus, additional regular layers on the cell surface are formed by halotolerant alkaliphilic methanotrophs of the genus *Methylomicrobium*. At the same time, three major types of supramolecular organization of S-layers represented as cup-shaped structures, fine inverted cones, or protein subunits forming a planar *p*2 symmetry are present in different strains of the species *Mm. alcaliphilum*. It is notable that the character of supramolecular organization of S-layers in *Mm. bury-*

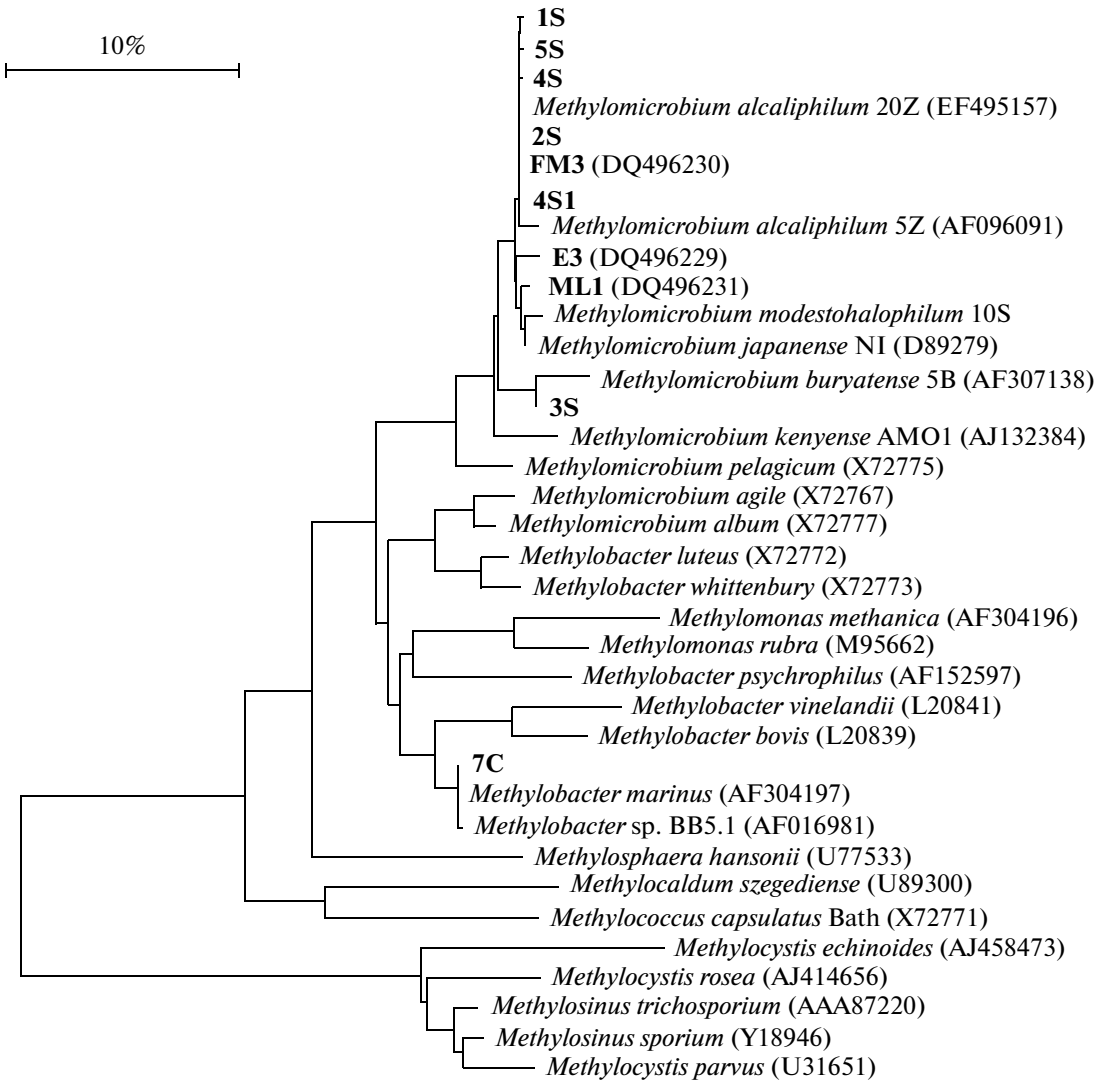


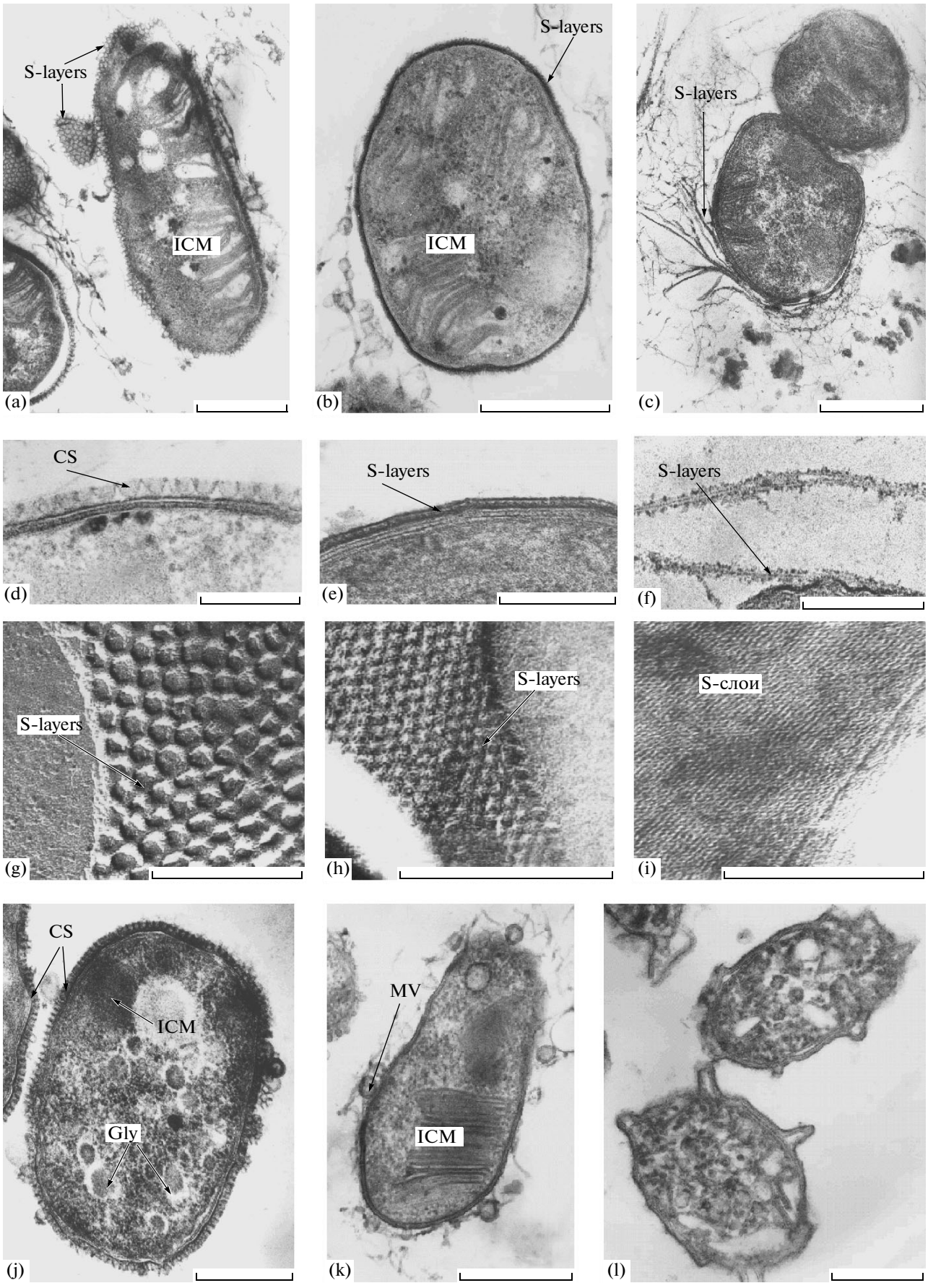
Fig. 3. Phylogenetic tree constructed on the basis of comparative analysis of nucleotide sequences of the 16S rRNA gene of the new isolates and other methanotrophic bacteria. The tree was constructed relative to the 16S rDNA sequence of *Bacillus subtilis*. New strains are in bold.

atense 3S, which is slightly different from that of *Mm. alcaliphilum*, is analogous to other strains of this species [3].

The functions of the S-layers typical for many bacteria have yet to be ascertained [19]. For halotolerant alkaliphilic methanotrophs, one can only suggest the role of S-layers as an additional rigid framework of the cell wall, which prevents cell lysis under the conditions of changing osmotic pressure of soda lakes, which are liable to drying up and fluctuations of salinity. At the same time, the absence of S-layers in the neutrophilic halophilic isolates referred to the genera *Methylobacter* and *Methylohalobius* does not allow us to consider these surface structures as a universal mechanism facilitating survival under high salinity. Elucidation of the functions of S-layers in methanotrophs depends, first of all, on successful decoding of their genetic determinants [7].

Osmoprotectants and analysis of the genes of ectoine synthesis. During the growth under conditions of

Fig. 4. Cell morphology and ultrastructure of the halo(alkali)philic methanotrophs *Methylomicrobium alcaliphilum* 1S (a, d, g), *Methylomicrobium alcaliphilum* 5S (b, e, h), *Methylomicrobium alcaliphilum* ML1 (c, f, i), *Methylomicrobium buryatense* 3S (j), *Methylobacter marinus* 7C (k), and *Methylohalobius crimeensis* 3C (l). Ultrathin sections (a–f, j–l); cell surface cryofractures showing *p*6 symmetry of S-layers (g, h); negatively stained S-layer with *p*2 symmetry (i). Scale bar: 0.5 μm (a–c, j–l) and 0.2 μm (d–i). Gly, glycogen granules; CS, cup-shaped structures; ICM, intracytoplasmic membranes; MV, membrane vesicles.


S-layers
ICM
(a)
S-layers
ICM
(b)
S-layers
(c)
CS
(d)
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(e)
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S-layers
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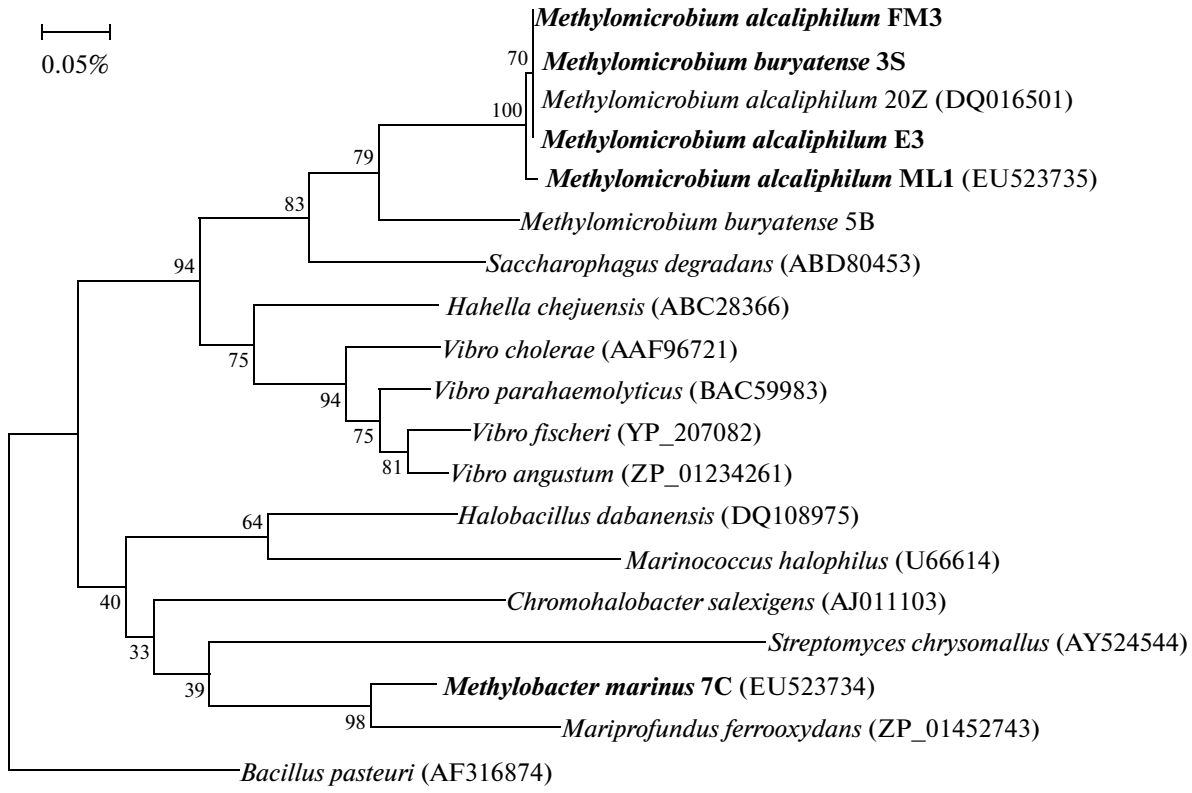

Fig. 5. Phylogenetic tree constructed on the basis of comparative analysis of translated amino acid sequences of the *ectC* gene of methanotrophic isolates and some halophilic and halotolerant eubacteria. The tree was constructed relative to the EctC fragment of *Bacillus pasteurii*.

high salinity (4–6% NaCl), the isolated methanotrophs accumulated ectoine (50–80 mg/g dry cells), glutamate (25–55 mg/g), and sucrose (30–60 mg/g). Thus, halophilic and halotolerant methanotrophs, irrespective of their taxonomic affiliation, synthesize the same spectrum of osmoprotectants to equilibrate the external osmotic pressure [7–9].

A DNA fragment including the 3'-terminal sequence of the *ectB* gene and the 5'-terminal region of the *ectC* gene was amplified from the DNA of five strains of methanotrophic isolates and *Mm. buryatense* $5B^T$ by using the previously developed degenerate primers [11]. The sequencing of the PCR products showed that these incomplete open reading frames were oriented in the same direction, indicating possible arrangement of the genes of ectoine synthesis in a single cluster. Phylogenetic analysis of the translated amino acid sequences of the *ectC* gene fragment (261 bp, which makes up two-thirds of its complete sequence) showed that ectoine synthase of the isolated methanotrophs exhibited a homology with EctC from other halophilic bacteria (Fig. 5) and the methanotroph *Mm. alcaliphilum* 20Z (50–100%), thus confirming rather high conservation of the ectoine synthesis pathway in bacteria [20].

Based on analysis of the EctC sequences and taxonomic and ecogeographical grouping of the methanotrophic isolates, some patterns may be established. For example, in different strains of *Mm. alcaliphilum* (FM3, E3, ML1, and 20Z) and in *Mm. buryatense* 3S isolated from silt of the soda lakes of Mongolia, Egypt, North America, and Russia, respectively, the translated amino acid sequences of the *ectC* gene were practically identical (98–100%) (Fig. 5). On the other hand, the *ectC* genes proved to be rather divergent in two strains of the same species, *Mm. buryatense* 5B and 3S (81%), which differed also in the upper limit of halotolerance (6 or 9% NaCl, respectively). It excludes neither the lateral transfer of the *ect* genes nor the high rate of their divergence in methanotrophs. Moreover, ectoine and the genes of its biosynthesis have been found for the first time in the neutrophilic, moderately halophilic *Mb. marinus* 7C. At the same time, the high divergence between EctC of *Mb. marinus* 7C and methanotrophs of the genus *Methylomicrobium* (~50% of identity) and the clustering of EctC of *Mb. marinus* 7C with the enzyme from the marine halophilic bacterium *Mariprofundus ferrooxydans* (78.5%) imply horizontal transfer of the *ect* genes.

Thus, methanotrophs adapted to the conditions of hypersaline and soda lakes are represented by halo-

Table 3. Fatty acid profile in the cells of new isolates (% of the total content)

Acid	5S		4S pH 9.5	7C pH 7.5
	pH 9.5	pH 7.2		
14:0	2.1	3.4	2.6	7.5
15:0	3.0	2.5	1.8	3.9
16:1ω7c	49.8	52.0	49.5	53.2
16:1ω7t	9.3	10.0	8.6	5.1
16:1ω5c	18.0	14.7	19.1	16.3
16:0	16.1	15.4	16.8	11.3
17:1ω8c	0.3	0.2	0.1	0.4
3h16:0	0.1	0.1	0.1	0.4
18:2		0.1		0.5
18:1ω9	0.5	0.3	0.2	0.5
18:1ω7	0.3	0.4	0.4	0.7
18:0	0.5	0.9	0.7	0.3

philic or halotolerant, facultatively alkaliphilic methanotrophs of morphotype I. These bacteria assimilate methane carbon via the RuMP cycle, the most energetically efficient pathway of assimilation of C_1 substrates, which gives advantages in osmoadaptation, in particular, maintenance of the ionic cell homeostasis, the synthesis of osmoprotectants and/or S-layers. Comparative analysis of the *ectC* genes indicates the horizontal transfer of the genes of the pathway of synthesis of the osmoprotectant ectoine as a quite probable way for methanotrophs to become resistant to high salinity.

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