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REVIEW PAPERS

# Moderately Haloalkaliphilic Aerobic Methylobacteria

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**Abstract**—Aerobic methylobacteria utilizing oxidized and substituted methane derivatives as carbon and energy sources are widespread in nature and involved in the global carbon cycle, being a unique biofilter on the path of these $C_1$ compounds from different ecosystems to the atmosphere. New data on the biological features of moderately halophilic, neutrophilic, and alkaliphilic methylobacteria isolated from biotopes with higher osmolarity (seas, saline and soda lakes, saline soils, and deteriorating marble) are reviewed. Particular attention is paid to the latest advances in the study of the mechanisms of osmoadaptation of aerobic moderately haloalkaliphilic methylobacteria: formation of osmolytes, in particular, molecular and genetic aspects of biosynthesis of the universal bioprotectant ectoine. The prospects for further studies of the physiological and biochemical principles of haloalkalophily and for the application of haloalkaliphilic aerobic methylobacteria in biosynthesis and biodegradation are discussed.

*Key words*: aerobic methylobacteria, halo(alkali)philes, taxonomy, phylogeny, ecophysiology and osmoadaptation, bioremediation.

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Conventionally, moderate halophiles are considered to be microorganisms with optimal growth at 3–15% NaCl in the medium [1, 2]. This is a heterogeneous physiological group of microorganisms belonging to different classes of prokaryotes and eukaryotes. Before 1980, only six moderately halophilic (nonmethylotrophic) bacterial species had been described and included in Approved Lists of Bacterial Names [3]. However, several dozen species of moderately halophilic bacteria from different genera are currently known [2, 4]. Marine aerobic methylobacteria of the genus *Methylophaga* were the first among the halophilic methylotrophic isolates [5–8]. Later, the broad distribution of moderately halophilic methylobacteria in different biotopes was demonstrated, and the representatives of novel taxa were described.

The ability of bacteria to grow or survive in saline and alkaline media is of great ecological, industrial, and epidemiological importance because haloalkalophilic/tolerant organisms cause damage to materials and foodstuffs, as well as certain diseases in humans and animals [9]. Saline and alkaline habitats occur on all continents and are populated by microbial communities which are adapted to these biotopes. The interest in haloalkaliphilic/tolerant microorganisms is caused not only by the necessity for understanding the mechanisms of adaptation to high salinity and pH values, but also by the prospects for their application in biotechnology. Many methylobacteria obtained in pure cultures are neutrophiles. However, the spectrum of the known halophilic and alkaliphilic methylobacteria has been substantially broadened recently; it has been ascertained that they are involved in the global carbon cycle in saline and alkaline biotopes. Haloalkaliphilic methylobacteria, being organisms with minimal growth requirements, are convenient objects for the study of osmoadaptation mechanisms. The biological features of moderately halophilic, neutrophilic, and alkaliphilic methylobacteria are considered below.

## ECOLOGY OF HALOPHILIC METHYLOBACTERIA

**Marine ecosystems.** Methanol, methylated amines, chloro-, bromo-, and iodomethane, dimethyl sulfoxide, methanethiol, methanesulfonic acid, and other $C_1$ compounds are present in marine ecosystems; they are formed by anaerobic microorganisms and algae and also enter these biotopes with precipitation.

The first halophilic methylobacteria were isolated from marine samples; they belonged to the genus *Methylophaga*: *M. marina*, *M. thalassica* [8], *M. sulfidovorans* [10], and *M. limanica* [11]. The widespread occurrence of *Methylophaga* bacteria in marine sam-

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ples has been shown recently by in situ hybridization with 16S rRNA-specific fluorescent-labeled oligonucleotide probes (FISH). For example, in situ hybridization with a MPH-730 probe specific for *Methylophaga* revealed the presence of methylobacteria of this genus in all ten analyzed samples of coastal marine sediments from France. Moreover, the cells of *M. marina* were revealed in five samples with another species-specific probe, MPH-994 [12]. However, the quantification of *Methylophaga* bacteria in marine samples has not been performed.

From green algae *Ulva lactuca* and *Cystoseira trinodes* collected in different regions of the Red Sea littoral zones, we have also isolated methylobacteria, which were identified as strains (KM) of the species *M. marina* by methods of polyphasic taxonomy, including 16S rRNA gene sequencing (99% similarity). Vitamin $B_{12}$ auxotrophy has been considered one of the typical characteristics of the *Methylophaga* genus. An essential distinction of the two new *M. marina* isolates, KM3 and KM5, was the absence of dependence on growth factors, including vitamin $B_{12}$ [13]; this finding reduces this feature to the phenotypic level.

The neutrophilic, moderately halophilic methylobacterial culture isolated from the firth of the Sea of Azov was identified as a member of the new genus *Methylarcula marina* [14]. The new genus *Marinosulfonomonas* was also proposed for the strains of methylotrophic bacteria isolated from the water samples of the Plymouth Strait (English Channel) and represented by the species "*Marinosulfonomonas methylotropha*" [15, 16].

Moderately haloalkaliphilic *Paracoccus haeundaensis* sp. nov. has been isolated from the Sea of Japan [17] and the facultative neutrophilic methylotroph *Leisingera methylohalidivorans*, from Californian coastal seawater [18].

Thus, according to the few publications available, moderately halophilic methylobacteria of the genus *Methylophaga* are the most widespread in seawater. It may be assumed that the biodiversity of methylobacteria from marine ecosystems extends further.

**Soda lakes.** Microbial communities of epicontinental soda lakes are considered as analogues of relict centers of terrestrial microbiota diversity [19, 20]. It has been established that the microbial communities of alkaline water bodies are a multidimensional cooperative system with stable trophic links and the nearly closed cycle of matter [21]. Consequently, these water bodies are suitable for studying the phylogeny, biodiversity, and survival mechanisms of prokaryotes exposed to quite a number of stress factors, primarily to significant fluctuations in temperature, pH, NaCl concentration, and total mineralization of the medium.

The athalassic lakes of the African Rift, Central Asia, South-West Siberia, Southern Transbaikalia, and North America have been actively studied in the last decade. Comprehensive studies of these ecosystems have revealed different physiological groups of (halo)alkaliphilic prokaryotes: cyanobacteria, acetogens, sulfate-reducing bacteria, phototrophs, hydrogen and thionic bacteria, natronobacteria, spirochetes, and alkaliphilic thermotogae [2, 21–28], as well as aerobic methanotrophs [29]. Various $C_1$ compounds are known to be formed in soda lakes: methanol, formaldehyde, and formate as intermediates of methane oxidation by methanotrophs [30] and methylated amines as products of betaine degradation by methanogens [21]. In addition, some $C_1$ compounds are drawn from the atmosphere with rain and snow, e.g., methanesulfonic acid [31]. It is logical to think that aerobic methylobacteria may also be components of the alkaliphilic microbial communities of soda lakes.

Indeed, from 35 samples taken from alkaline lakes of Southern Transbaikalia and Mongolia, bacterial enrichment cultures were obtained by us which actively grew on methanol as a carbon and energy source at 3–9% NaCl and pH 9–10 and did not require additional growth factors. These associative cultures were rather stable and have been maintained for many years at 4°C in a liquid medium, with re-inoculations once a year. Upon drying, the culture medium became jellylike due to accumulation of an exopolysaccharide (EPS), obviously stabilizing the cells [32].

Isolation of pure methylobacterial cultures from such haloalkaliphilic consortia was complicated by the fact that single colonies on agarized media were represented by several morphotypes of cells, i.e., they were associations of different bacterial species. Assuming that monocultures required growth factors provided by the satellites, we used agarized media with addition of the culture liquid of methanotrophs which had been isolated previously from the same soda lake sludge samples. As a result, we succeeded in isolation of 15 pure cultures of gram-negative methylobacteria. Most of them were auxotrophic by vitamin $B_{12}$, and two isolates were in need of biotin.

$B_{12}$-dependent strains of the genus *Methylophaga* were represented by small motile monotrichous rods. The cells were characterized by a vast periplasmic space (~20 nm). Cells with similar ultrastructure were present in all associative methylotrophic cultures isolated from sludge samples of different soda lakes. Two $B_{12}$-dependent isolates were identified as the novel alkaliphilic species of the genus *Methylophaga*: *M. alcalica* and *M. natronica* [33, 34]. On the contrary, two biotin-dependent halotolerant, facultatively methylotrophic isolates were classified as a novel species *Ancylobacter natronum* [35]. Methanotrophs have been previously isolated from the same sludge samples [29]. Apparently, this trophic connection is determined by the fact that the intermediates of methane oxidation

(methanol, formaldehyde, and formate) are partially excreted by methanotrophs into the medium. In addition, many strains of the genus *Ancylobacter*, being oligotrophs, can utilize hydrogen as an electron donor and $CO_2$ as a carbon source. Such metabolic flexibility allows these chemolithotrophs to coexist with true methylotrophs in extreme biotopes [36]. Interestingly, Namsarayev and Zavarzin have also isolated *Ancylobacter aquaticus* Z-238 and Z-2434 (previously *Microcyclus aquaticus*) from the enrichment culture of methanotrophic bacteria obtained from a lowland bog [37].

Thus, these findings allowed us to consider aerobic methylobacteria as a functional component of the microbial communities of soda lakes and supplemented the previously proposed schemes of trophic relationships between (halo)alkaliphilic heterotrophs and oligotrophs, methanogens, and methanotrophs [19, 21, 29]. Presumably, aerobic methylobacteria, being an indispensable link in the trophic chain of the microbiota of alkaline ecosystems, provide the return of carbon from the products of incomplete oxidation of methane and methylamines to the total pool of organic matter of these water bodies (Fig. 1).

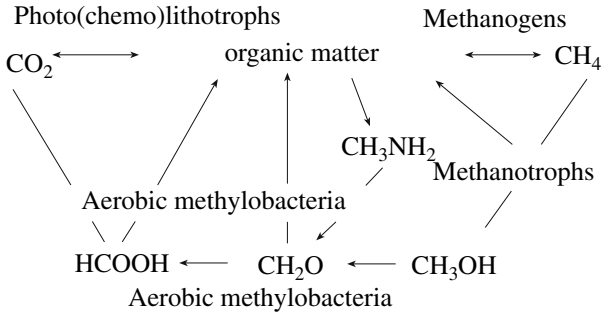


**Fig. 1.** Aerobic methylobacteria as components of the microbial trophic chain of the methane cycle in soda and saline lakes.

**Soils and weathering rocks.** Although $C_1$ compounds are widespread in nature, the methylotrophs of saline soils have not been studied. Only one report on the isolation of halophilic methylobacteria *Methylarcula terricola* from soils with high contents of NaCl has been published [14].

In addition to being vulnerable to the influence of physicochemical factors, stone monuments are susceptible to destruction by microbial communities of biofilms that comprise bacteria, fungi, and algae [38, 39]. Trophic relationships have been shown to exist between the microorganisms of biofilms on a stone surface. The greatest variation in microflora is observed on the surface of deteriorated marble. Individual representatives of this microbiota are characterized by high resistance to unfavorable external factors, the ability to penetrate into the substrate, and to develop on marble for a long time, causing its gradual destruction [38, 39].

The destructive effect of microorganisms on marble is due to the formation and excretion of organic and inorganic acids, enzymes, pigments, and polysaccharides catalyzing the processes of marble destruction. In addition, microorganisms penetrating into marble change its strength properties. The formation of biological deposits on the surface of marble monuments is most often due to the presence of sources of organic (e.g., closeness of green plantations) and atmospheric pollutions. Methanotrophs of the genera *Methylocystis* and *Methylosinus* have been found previously on deteriorating marble [40]. Though the atmospheric content of methane (up to 1.7 ppmv) is insufficient for the growth of most methanotrophs [41], some members of the genus *Methylocystis* are able to grow at such low $CH_4$ concentrations [42]. It is assumed that the local concentration of methane in the stone is higher, which is usually associated with methanogenesis as a result of degradation of organic compounds by different microorganisms [40, 43].

In addition, a sensational report has appeared recently that plants form methane by an unknown biochemical process different from archaeal methanogenesis. According to preliminary estimates, living plants produce 60 to $240 \times 10^6$ tons of $CH_4$ carbon per year; another 0.5 to $7 \times 10^6$ tons of $CH_4$ carbon is produced by fallen leaves. This totals 10–30% of the total annual inflow of methane into the atmosphere (>500 mln tons of C), including from technogenic sources [44]. Obviously, close to green plantations, the $CH_4$ concentration is higher than the atmospheric mean value. It has been also proved experimentally that plants form methanol as a result of the action of intracellular pectinmethyl esterases and lignin demethylases, being global suppliers of atmospheric $CH_3OH$: over 100 mln tons C/year [45–47]. Probably, these methane and methanol sources cause the development of the aerobic methylotrophic bacteria comprising biofilms on stones.

We have obtained six enrichment cultures and five pure cultures of aerobic methylobacteria from the scrapings taken from disintegrating parts of marble monuments in the Moscow Kremlin (pH of the marble surface in the place of sampling was 8.7–9.1). The pure cultures proved to be identical in morphology, physiological, and biochemical properties; they had DNA–DNA homology of 98–99%. A representative of this group of haloalkaliphilic methylobacteria has been described as a novel species, *Methylophaga murata* [48]. It is able to produce formic acid (4–10 mM) from methanol and excrete; this may be one of the factors causing marble deterioration. Consequently, aerobic methylobacteria, as a functional component of oligotrophic communities (biofilms) on the surface of stones, are involved in their destruction and intensify the processes of physicochemical erosion.

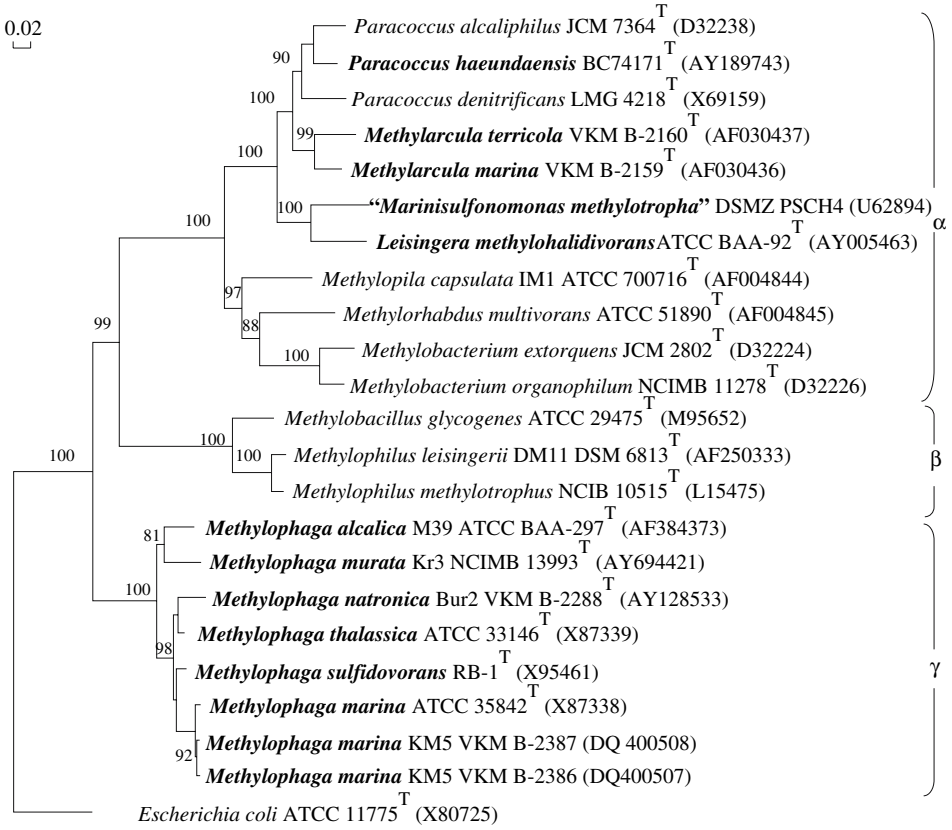


**Fig. 2.** Phylogenetic position of haloalkaliphilic aerobic methylobacteria. The scale corresponds to 2 nucleotide substitutions per every 100 nucleotides. Numbers indicate statistical reliability of branching order determined by bootstrap analysis.

## TAXONOMY, PHYSIOLOGICAL, AND BIOCHEMICAL FEATURES OF HALOALKALIPHILIC METHYLOBACTERIA

All currently known haloalkaliphilic methylobacteria belong to *Alpha-* and *Gammaproteobacteria* (Fig. 2).

***Ancylobacter natronum*** isolated from soda lakes belongs to *Alphaproteobacteria.* It is represented by colorless, asporogenous, nonmotile, ovoid rods growing in a wide temperature range of 5–42°C, pH 6.5–9.0, with the optimum at 25–29°C, pH 8.0–8.5, and 0.5–0.75% NaCl. Though its growth rate on the medium with methanol significantly decreases at levels as low as 2% NaCl, very slow growth is still possible in the presence of 6% NaCl. It employs the ribulose bisphosphate (RBP) cycle [35].

Moderately halophilic methylobacteria of the genus *Methylophaga* grow in a wide range of NaCl concentrations (0.5–20%) and require $Na^+$ ions; many species are auxotrophic by vitamin $B_{12}$. However, the isolates from the Red Sea algae classified as novel strains of *M. marina* do not depend on vitamin $B_{12}$ or other growth factors [13]. At the same time, *M. marina, M. thalassica, M. limanica, M. natronica*, and *M. murata* are restricted facultative methylotrophs (i.e., they grow on glucose or fructose, methanol, and methylamine), whereas *M. alcalica* and *M. sulfidovorans* are obligate methylotrophs growing only on $C_1$ substrates. *M. sulfidovorans* grows in a liquid culture on methanol, methylamine, and methanesulfonic acid but does not grow on agarized media. Alkaliphilic species *M. alcalica, M. natronica*, and *M. murata* are able to grow at lower temperatures (0–4°C) than neutrophilic *M. marina, M. thalassica, M. limanica*, and *M. sulfidovorans.*

Methylobacteria of the genus *Methylophaga* with the RMP pathway possess the Krebs cycle opened at the level of α-ketoglutarate dehydrogenase and comparatively low DNA G+C content (43–49 mol %); they contain $Q_8$ as the major ubiquinone, $C_{16:0}$ and $C_{16:1}$ fatty acids; EPS are produced under nitrogen or phosphorus deficiency in the medium. In contrast to neutrophiles, the predominant fatty acid in alkaliphilic species is also $C_{18:1}$ [13, 33, 34, 48, 49]. The genus *Methylophaga* belongs to *Gammaproteobacteria* [50].

Facultative methylobacteria of the genus *Methylarcula* optimally grow on methylamine at weakly alkaline pH values and NaCl content of 3–8%. The cells are nonpigmented short nonmotile rods which produce intracellular poly-β-hydroxybutyrate. They possess the

isocitrate lyase negative (icl$^-$) variant of the serine pathway, have the complete Krebs cycle, and DNA G+C content of 57–61 mol %; the major ubiquinone is $Q_{10}$; the main fatty acids are $C_{18:0}$, $C_{18:1}$, and $C_{\Delta 19:0}$. The genus *Methylarcula* belongs to the *Alphaproteobacteria*.

The novel facultative methylotroph *Leisingera methylohalidivorans* utilizes halomethanes ($CH_3Br$, $CH_3Cl$, and $CH_3I$) as carbon and energy sources [18]. It grows poorly on dimethyl sulfide but does not utilize methylated amines, methanol, or formate as a carbon and energy source. The cells are nonpigmented motile gram-negative rods, pleomorphic when growing on media with yeast extract or glycine betaine; optimal growth is at 3–4% NaCl, pH 7.7, 27°C; they belong to the *Alphaproteobacteria.* Although the genus *Leisingera* has been validated, its chemotaxonomic characteristics and $C_1$ metabolic pathway have not been described [18].

The genus *"Marinosulfonomonas"* includes *"Marinosulfonomonas methylotropha,"* which utilizes methanesulfonic acid, methanol, methylammonium chloride, and various polycarbon substrates. Optimal growth is at pH 7.4, 3% NaCl, 30°C. The cells are nonmotile rods, single or gathered in rosettes of 5–30 cells in liquid culture. Growth is very weak on solid medium but good in liquid medium. Possesses the serine pathway, belongs to the *Alphaproteobacteria*.

The common and differentiating characteristics of the best studied taxa of aerobic, moderately halo(alkali)philic/tolerant methylobacteria are summarized in table.

## PATHWAYS OF PRIMARY $C_1$ METABOLISM IN HALOALKALIPHILIC METHYLOBACTERIA

**Oxidation of methanol.** All known moderately haloalkaliphilic aerobic methylobacteria oxidize methanol to formaldehyde by means of the classical methanol dehydrogenase (MDH), the cofactor of which is pyrroloquinoline quinone. The enzyme has an optimum activity at pH 9.0, is activated by ammonium ions, and is an $\alpha_2\beta_2$ heterotetramer with subunit molecular masses (MM) of 62 and 8 kDa [51]. The *mxaF* gene encoding the major $\alpha$ subunit of MDH is very conservative; therefore the developed primers permit detection of this protein in methylotrophs of different taxonomic position. The role of MDH in the generation of metabolic energy in halo(alkali)philic methylobacteria becomes even more important due to their increased energy expenditure for the maintenance of intracellular ionic homeostasis. In addition, the periplasmic localization of MDH in halo(alkali)philic methylobacteria suggests that this enzyme must have acquired properties that would allow it to function under high osmolarity of the medium.

Two forms of methanol dehydrogenase, MDH1 and MDH2, have recently been found in the marine methylobacterium *Methylophaga* sp. 1 and characterized. These forms have virtually identical catalytic properties but differ in their total charges and electrophoretic mobility [52]. On the other hand, only one copy of the MDH gene has been found in the chromosome of the nonhalophilic *Methylobacterium extorquens* AM1 [53–55]. The question of whether the occurrence of two MDH isoforms in *Methylophaga* bacteria is associated with their ability to grow at high salinity remains to be elucidated.

**Oxidation of methylamine.** Halophilic methylobacteria oxidize methylamine mainly through the *N*-methylglutamate pathway, though some species of the genus *Methylophaga* concurrently possess a methylamine dehydrogenase [8, 11]. The products of short-term fixation of $^{14}CH_3NH_2$ in bacteria of the genera *Methylarcula* and *Methylophaga* are *N*-methyl derivatives of glutamate: γ-glutamylmethylamide (γ-GMA) and *N*-methylglutamate (*N*-MG). Enzymological studies have revealed the enzymes responsible for the synthesis and degradation of these compounds in halophilic methylobacteria: γ-GMA synthetase, *N*-MG synthase, γ-GMA, and *N*-MG lyases, which degrade *N*-methyl derivatives of glutamate to formaldehyde and $NH_4^+$ with regeneration of glutamate (the primary acceptor of methylamine). On the contrary, γ-GMA lyase has not been found in nonhalophilic methylobacteria with the *N*-methylglutamate pathway of methylamine oxidation [11, 56].

The γ-GMA lyase from *Methylophaga* sp. AA-30 has been shown to have two components. The high-molecular component (H) contains flavin and two subunits of 55 and 29 kDa and is apparently an $\alpha_4\beta_4$ heterooctameter of 360 kDa. On the other hand, the low-molecular 38 kDa polypeptide (L) contains one mole of thiol group per mole of protein [56]. It is known that the growth of nonhalophilic methylobacteria is usually inhibited by 1% NaCl. However, we have shown that the halotolerance of nonhalophilic methylobacteria with the *N*-methylglutamate pathway increases up to 3% NaCl in the presence of $CH_3NH_2$. At the same time, the members of the genera *Methylorhabdus*, *Methylopila*, and *Methylobacterium extorquens* AM1 using the direct pathway of methylamine oxidation involving amine dehydrogenase had similar halotolerance when grown on $CH_3OH$ and $CH_3NH_2$. It is assumed that glutamate (the primary acceptor of methylamine) and its *N*-methyl derivatives (*N*-MG and γ-GMA) possess osmoprotectant properties. Indeed, the addition of these compounds to the cultures of nonhalophilic methylobacteria growing on $CH_3OH$ increases their halotolerance up to 3% NaCl [57].

**Oxidation of dimethyl sulfide and methanesulfonic acid.** Ocean and sea water is saturated with

Differentiating characteristics of the best-studied moderately halo(alkali)philic aerobic methylobacteria

| Characteristic | *Methylarcula* gen. nov. spp. nov | | *Ancylobacter natronum* [35] | *Methylophaga* spp. nov. | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *M. marina* [14] | *M. terricola* [14] | | *M. marina* [8] | *M. thalassica* [8] | *M. limanica* [11] | *M. sulfidovorans* [10] | *M. alcalica* [33] | *M. natronica* [34] | *M. murata* [48] |
| Cell size, μm | 0.7 × 1.0 | 0.7 × 1.6–2.0 | 0.5 × 1.1–1.4 | 0.2 × 1.0 | 0.2 × 1.0 | 0.4 × 1.2 | 0.2 × 0.9 | 0.4 × 2.8 | 0.6 × 2.8 | 0.7 × 2.0 |
| Type of methylotrophy | facultative | | | restricted facultative | | | obligate | | restricted facultative | |
| Growth factors | – | – | biotin | $B_{12}$ | $B_{12}$ | $B_{12}$ | $B_{12}$ | $B_{12}$ | $B_{12}$ | $B_{12}$ |
| $C_1$ assimilation pathway | Serine | | RBP | Ribulose monophosphate | | | | Ribulose monophosphate | | |
| Predominant fatty acids | $C_{18:0}$, $C_{18:1}$, $C_{\Delta 19:0}$ | | $C_{18:1}$, $C_{\Delta 19:0}$ | $C_{16:0}$, $C_{16:1}$ | | | n/d | $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ | | |
| Major ubiquinone | $Q_{10}$ | $Q_{10}$ | $Q_{10}$ | $Q_8$ | $Q_8$ | $Q_8$ | $Q_8$ | $Q_8$ | $Q_8$ | $Q_8$ |
| Temperature range, °C (optimum) | 10–42 (20–35) | 10–40 (29–32) | 6–37 (25–29) | 10–40 (30–37) | 8–42 (30–37) | 10–40 (30–37) | 17–35 (22) | 4– 35 (25–29) | 4–37 (25–29) | 0–42 (20–32) |
| pH range for growth (optimum) | 5.0–10.5 (7.5–8.5) | 5.5–10.0 (7.5–8.5) | 6.5–9.5 (8.0–8.5) | 5.0–9.0 (7.0–7.5) | 5.0–9.0 (7.0–7.5) | 6.0–8.5 (6.7–7.2) | 6.0–9.0 (7.4–7.8) | 7.0–10.0 (9.0–9.5) | 7.0–11.0 (8.5–9.0) | 6.0–11.0 (8.0–9.0) |
| NaCl range, % (optimum) | 12 (3–8) | 14 (3–6) | 6 (0.5–0.75) | 12 (1 –4) | 12 (1–4) | 10 (3–6) | 12 (1.5–2.5) | 10 (3–4) | 10 (3–6) | 20 (3–9) |
| Osmoprotectants | Ectoine, glutamate | | – | Ectoine, glutamate | | n/d | n/d | Ectoine, glutamate, sucrose | | |
| DNA G+C content, mol% | 60.4 | 57.1 | 66.2 | 43.0 | 44.0 | 43.5 | 42.4 | 48.3 | 45.0 | 44.6 |
| Source of isolation | Firth, the Sea of Azov | Saline soil, Alushta | Soda lake, Transbaikalia | Seawater and sludge | | Firth, the Black Sea | Microbial mats, the Netherlands coast | Soda lake, Mongolia | Soda lake, Transbaikalia | Marble monument, Moscow Kremlin |
| Class of *Proteobacteria* | Alphaproteobacteria | | | Gammaproteobacteria | | | | | | |

Note: "–", absence of character; n/d, not determined.

dimethyl sulfide ($(CH_3)_2S$) which, as the product of degradation of dimethylsulfoniopropionate ($(CH_3)_2S^+CH_2CH_2COO^-$), is synthesized by many marine algae as an osmoprotectant [58, 59]. Global production of $(CH_3)_2S$ is evaluated as $50 \times 10^6$ tons S/yr. Several cultures capable of growing on dimethyl sulfide have been isolated from marine biotopes: moderately halophilic *Methylophaga sulfidovorans* [10] and three unidentified strains LIS 1–3 [60]. Oxidation of dimethyl sulfide to formaldehyde and methanethiol is catalyzed by monooxygenase in compliance with the equation:

$$(CH_3)_2S + НАДН + H^+ + O_2 \longrightarrow CH_2O + CH_3SH + H_2O + НАД^+.$$

Methanethiol is further transformed into formaldehyde and sulfide in another reaction:

$$CH_3SH + O_2 + H_2O \longrightarrow CH_2O + H_2S + H_2O_2.$$

It is interesting that *M. sulfidovorans* assimilates formaldehyde via the RMP pathway, whereas strains LIS 1–3 use the serine pathway.

A significant portion of dimethyl sulfide from oceans and seas enters the atmosphere and is exposed to photochemical oxidation to more stable compounds, methanesulfonic acid ($CH_2SO_3H$) and dimethylsulfone ($(CH_3)_2SO_2$), which return to aquatic and terrestrial biotopes in rain and snow. The analysis of Antarctic ice revealed huge reserves of methanesulfonic acid that had been accumulating for many thousands of years [61]. Moderately halophilic "*Marinosulfonomonas methylotropha*" was shown to possess a cytoplasmic monooxygenase degrading methanesulfonic acid through an unstable intermediate (hydroxymethane sulfonate) to formaldehyde and sulfite according to the equation

$$CH_2SO_3H + O_2 + НАДН + H^+ \longrightarrow CH_2O + НАД^+ + H_2SO_3 + H_2O.$$

Formaldehyde is further assimilated by this methylotroph via the serine pathway or oxidized to $CO_2$ and $H_2O$ with the formation of reduced equivalents necessary for the generation of metabolic energy and biosynthesis.

Cytoplasmic methanesulfonate monooxygenase was also found in the nonhalophilic methylotroph "*Methylosulfonomonas methylovora*" isolated from garden soil [15, 31]. Molecular analysis of the purified monooxygenase showed that the enzyme was a unique multicomponent complex [62–64]. A two-component hydroxylase MsmAB (43 and 21 kDa), an MsmC ferredoxine (16 kDa), and an MsmD reductase (38 kDa) were found. The corresponding structural genes *msmABCD* and their cluster on the chromosome were identified. The genes encoding the methylsulfonate transport system are located nearby. This system closely resembles the ABC type of transporters and includes a periplasmic substrate-binding protein MsmE (42 kDa), an integral membrane-associated protein MsmF (29kDa), an ATP-binding protein MsmG (31 kDa), and a permease MsmH (32 kDa). The *msmABCD* and *msmEFGH* genes were shown to form two different operons corresponding to the oxygenase and transport systems. At the same time, methanesulfonate induced expression of the *msmABCD* operon, while the *msmEFGH* operon was expressed constitutively, i.e., in "*M. methylovora*" grown on the medium with methanol [65].

Halophilic aerobic methylobacteria seem to play an important part in degradation of dimethyl sulfide and methanesulfonate in marine environments, and nonhalophilic members of the genera *Hyphomicrobium, Methylobacterium*, and *Pedomicrobium*, in fresh waters and soils [66].

## OSMOADAPTATION OF HALOALKALIPHILIC AEROBIC METHYLOBACTERIA

It was shown that the content of negatively charged phosphatidyl glycerol and cardiolipin in the phospholipid pool of *M. murata* increased as the growth medium salinity became higher, while the content of phosphatidyl ethanolamine decreased [48]. Previously, such an effect had been reported for gram-negative halophilic and halotolerant eubacteria [67], including methanotrophs [68]. Obviously, the quantitative changes of the phospholipid composition at high osmolarity of the medium are caused by the need for additional stabilization of cell membranes.

As is well known, two strategies of maintenance and regulation of osmotic balance operate in the microbial world [4, 69]. The first is the strategy of selective accumulation of inorganic ions in the cytoplasm (the so-called "saline" type of osmoadaptation), which requires additional adaptation of cell organelles and of the whole enzymatic system. The second strategy (equilibration of the ambient osmotic pressure) is associated with accumulation of low-molecular organic compounds which do not affect the physiological functions of a cell (the strategy of "compatible solutes"). Such osmoadaptation does not require essential structural and functional changes of the cells, thus providing more flexible and reliable adaptation to osmotic fluctuations.

It should be noted that many osmoprotectants accumulated by cells in molar concentrations are responsible for osmotic balance and, at the same time, metabolically inert [70]. One of these is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine-carboxylic acid), discovered by E. Galinsky as a minor osmoprotectant in a halophilic phototroph *Ectothiorhodospira halochloris* [71]. Since then, ectoine has been found in a number of heterotrophic halotolerant bacteria of the genera

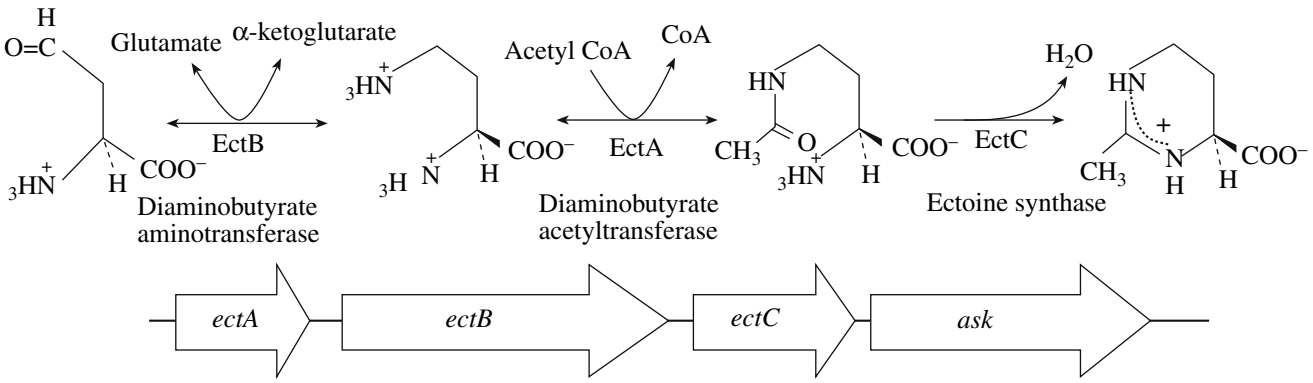


**Fig. 3.** Organization of the genes of the ectoine biosynthesis pathway in halo(alkali)philic aerobic methylobacteria: *ectA*, DAB acetyltransferase; *ectB*, aminotransferase; *ectC*, ectoinesynthase; *ask*, aspartokinase.

*Brevibacterium, Nocardiopsis, Streptomyces, Bacillus, Marinococcus, Sporosarcina, Chromohalobacter, Halomonas, Vibrio* [72–77], and in obligate methanotrophs of the genus *Methylomicrobium* [78].

We have revealed that methylobacteria of the genera *Methylarcula* and *Methylophaga* synthesize two basic osmoprotectants: glutamate and ectoine, while the members of the genus *Methylophaga* also accumulate sucrose in minor quantities [14, 33, 34]. The cells of methylobacteria of the genus *Methylophaga* grown at low NaCl concentrations (2–3%) were shown to contain glutamate alone, while ectoine, glutamate, and sucrose were accumulated when the salinity of the medium was increased to 10–12%. The total content of these osmoprotectants in the strains under study, per amount of intracellular water, completely balanced the medium osmolarity. In addition, the concentration of the above-mentioned osmoprotectants in *M. murata* increased as the temperature of cultivation became lower. Thus, the intracellular content of ectoine at 4°C was two times higher than at 29°C. It is notable that *M. murata* cells grown at 6–9% NaCl survived after heating at 70°C for 15–20 min, sustained multiple cycles of freeze–thawing, and tolerated lyophilization without the addition of cryoprotectants, which was probably due to a high level of ectoine accumulation.

Accumulation of polyhydroxybutyrate by bacteria of the genus *Methylarcula* was apparently the result of the operation of the serine pathway, which includes transformations of organic acids and amino acids, but not of phosphosugars as in the RMP cycle. On the other hand, bacteria of the genus *Methylophaga* synthesize sucrose from UDP-glucose and fructose-6-phosphate; this process involves sucrose phosphate synthase/phosphatase. Consequently, sucrose precursors are formed in the primary reactions of the RMP cycle.

The pathway of ectoine biosynthesis has been studied in detail in three species of moderately halophilic heterotrophic eubacteria: gram-positive *Marinococcus halophilus* [74], gram-negative *Halomonas elongata* [79], and *Chromohalobacter salexigens* [80] growing on complex nutrient media with glucose, and in the obligate methanotroph *Methylomicrobium alcaliphilum* 20Z [81–83]. We have shown that methylobacteria of the genera *Methylophaga* and *Methylarcula* growing on $C_1$ substrates in the presence of NaCl also use this pathway of ectoine biosynthesis, since they possess high activities of aspartokinase, aspartate-semialdehyde dehydrogenase, 2,4-diaminobutyrate (DAB) aminotransferase (EctB), DAB-acetyltransferase (EctA), and ectoine synthase (EctC) (Fig. 3).

Based on the analysis of EctA, EctB, and EctC amino acid sequences deposited in GenBank, we have developed a system of oligonucleotide primers to be used for decoding of the structure and order of location of the *ectA*, *ectB* and *ectC* genes encoding the enzymes of ectoine biosyntehsis (DAB-acetyltransferase, DAB-aminotransferase, and ectoine synthase, respectively) in *Methylophaga alcalica* M8 and *Methylophaga thalassica* MT. Analysis of the translated nucleotide sequences of the *ectABC* cluster has shown that their products are the proteins of 172, 443, and 134 amino acid residues (19.0, 48.7, and 15.3 kDA, respectively) in *M. alcalica* M8 and the proteins of 169, 445, and 134 amino acid residues (18.8, 48.9, and 15.4 kDA, respectively) in *M. thalassica* MT.

The phylogenetic analysis of amino acid sequences of the genes/enzymes of ectoine synthesis in these methylobacteria showed high homology (75–85%) with the corresponding sequences of haloalkaliphilic/tolerant methanotrophs and only 36–63% similarity to those of other halophiles. Besides, *Methylophaga alcalica* M8 and *Methylophaga thalassica* MT, like methanotroph *Methylomicrobium alcaliphilum* 20Z, were shown to differ in the organization of the genes of ectoine biosynthesis, as compared to heterotrophic halophiles. For example, an additional aspartokinase gene adjoins the *ectABC* genes [81, 82], which suggests the presence of a specific aspartokinase isoform in methylobacteria (Fig. 3). This feature may provide the

control of the synthesis of the ectoine precursor, aspartyl phosphate, which is relatively independent of the main constructive metabolism. At the same time, a significant divergence of the *ect* genes was revealed in methylotrophic and heterotrophic prokaryotes [82].

The *ectA* gene encoding DAB-acetyltransferase in *M. alcalica* M8 has been cloned and expressed in *Escherichia coli.* It has been shown that DAB-acetyltransferase is a homodimer of 40 kDa. The enzyme is the most active at pH 9.5 and 32–34°C; its activity increases appreciably in the presence of 250 mkM KCl. The Michaelis constants of the recombinant DAB-acetyltransferase are 465 and 32.7 μM for L-2,5-DAB and acetyl CoA, respectively.

Thus, aerobic methylobacteria of the genera *Methylophaga* and *Methylarcula* accumulating ectoine (up to 20% of dry biomass) are well adapted to existence in extreme ecosystems, characterized by drastic fluctuations in the temperature and salinity of the medium.

## CONCLUSIONS

In the last decade, a concept has been formulated of the taxonomic and structural and functional diversity of moderately halophilic aerobic methylobacteria and their important ecophysiological role in different biotopes.

Aerobic moderately halo(alkali)philic methylobacteria have been found in seawater, soda lakes, saline soils, and deteriorating marble. Usually, these methylobacteria are associated with methanotrophs because they utilize the products of incomplete methane oxidation—methanol, formaldehyde, and formic acid. In addition, halo(alkali)philic methylobacteria form stable methylotrophic communities with heterotrophs, supply exometabolites to the latter and, in turn, obtain growth factors (vitamins).

It has been shown that one of the mechanisms of osmoadaptation in halophilic/tolerant methylobacteria is the de novo synthesis and preferential intracellular accumulation of ectoine, a cyclic imino acid with a high water-retaining capacity. Usually, halo(alkali)philic methylobacteria synthesize ectoine only in conditions of high salt contents (≥3% NaCl), when energy expenditure on the maintenance of ionic gradients increases and the energy status of cells decreases [33, 34].

The interest in bioprotectants (ectoines, beatines) is in many respects determined by their potential application as "chemical chaperons" having a stabilizing and protective effect on enzyme systems and on DNA- and RNA-protein complexes [84]. Ectoine and hydroxyectoine are of the greatest interest since they are highly effective bioprotectants in the cryopreservation of genetic material and cell lyophilization; they are applied in biochemical studies as enzyme stabilizers and in cosmetics as water-retaining reagents, which easily penetrate into the cells and increase their turgor. It is not surprising that methylobacteria with high intracellular contents of ectoines can sustain heating, drying, and multiple cycles of freeze-thawing, and hence are quite adapted to continuously changing environmental conditions [48].

The recent progress in decoding of the four-gene cluster *ectABCask* in aerobic methylotrophs [82] has created real prerequisites for the construction of novel effective ectoine producers from methanol, which express these genes under the strong MDG promoter *mxaF.* At the same time, it is possible to construct strains that act as destructors of toxic $C_1$ compounds (e.g., halomethanes) at high mineralization and alkalinity of the medium.

Our findings pertinent to the structural organization of the *ect* genes in methylotrophs allow us to use the modern strategy for purification of enzymes of ectoine biosynthesis, based on cloning and affine chromatography, for detailed study of their properties and regulation. Moreover, the strains of halophilic neutrophilic and alkaliphilic methylobacteria available in our collection can be applied as model objects for elucidation of the characteristics of their energetic and constructive metabolism, including the principles of organization and regulation of ectoine biosynthesis at a molecular genetic level. In particular, comparative analysis of the structural organization and transcription regulation of the ectoine operon in neutrophil "serine" methylobacteria of the genus *Methylarcula* [14] will show the similarity to and difference from the previously studied haloalkaliphilic methanotrophs and methylobacteria with the RMP pathway.

Thus, osmoadaptation of haloalkaliphilic methylobacteria includes significant structural and functional changes (modification of the fatty acid and phospholipid membrane composition, intracellular accumulation of glutamate, sucrose, and $K^+$ in parallel with ectoine). The character of primary signal effectors and receptors initiating the cascade of responses to stress impacts of salinity and pH, including the bioenergetic patterns for the maintenance of the intracellular ionic homeostasis, has yet to be elucidated. It is obvious that the postulated mechanisms of osmoadaptation in haloalkaliphilic methylobacteria need further special studies by the methods of genomics and proteomics.

On the whole, recent progress in the study of the biology of haloalkaliphilic aerobic methylobacteria has brought us nearer not only to the understanding of the bases of their vital activity but also to the development of biotechnologies of ectoine synthesis from methanol and construction of new stress-polyresistant strains, destructors for bioremediation of extreme ecosystems from toxic $C_1$ compounds.

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