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REVIEW

Thermophilic and Thermotolerant Aerobic Methanotrophs

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Abstract—The review generalizes the modern data on the taxonomic, structural, and functional diversity of aerobic methanotrophs growing at 25–50°C (*Methylococcus capsulatus*), 30–62°C (*Methylocaldum szegediense, Methylocaldum gracile*, and *Methylocaldum tepidum*), and 50–65°C (*Methylothermus thermalis*), which belong mainly to the *Gammaproteobacteria.* The specific features of adaptation of these methanotrophs to the temperature influences are considered on the metabolic and genetic levels. The recent sensational reports on the discovery and primary characterization of thermoacidophilic methanotrophs of the phylum *Verrucomicrobia* surviving at extreme pH (1–2) and temperature (65°C) values, corresponding to extremely low levels of CH_4 and O_2 solubility, are analyzed. The possibilities of implementation of the biotechnological potential of thermophilic and thermotolerant methanotrophs are discussed.

Key words: aerobic methanotrophs, thermoadaptation, structural and functional features, taxonomy and phylogeny.

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The biotopes with permanently high temperatures are rather widespread on the Earth. High-temperature ecosystems such as active volcanoes, thermal springs, thermal water outlets, geysers, and wastes of some technological processes are inhabited by various physiological groups of thermophilic microorganisms. The intensive studies of the past two decades have drastically changed our notions about the taxonomic, structural, and functional diversity of thermophilic prokaryotes. In addition to isolation of thermophilic methanogens, carboxydobacteria, sulfate reducers, and sulfur- and iron-reducing prokaryotes from the thermal springs of Kunashir, Kamchatka (Russian Federation), the Yellowstone National Park (United States), and deepwater thermal vents of the Pacific Ocean [1–3], detection of aerobic methanotrophs in these ecotopes was reported.

Methane emission into the atmosphere is caused by an imbalance between the processes of CH_4 production and oxidation. Methane oxidation by the methanotrophic community ("bacterial gas filter") is undoubtedly the key biochemical process regulating the flux of this greenhouse gas. In the soil and freshwater ecotopes, methanotrophs utilize up to 80% of biogenic methane [4]. The processes of methane oxidation in high-temperature ecosystems are much less studied.

Since the hydrothermal fluids contain, apart from CO, CO_2, and H_2S, considerable amounts of CH_4, the presence of thermotolerant/thermophilic methanotrophs was suggested in these ecological zones. The solubility of gases in water is known to decrease with increasing temperature; this may limit the growth of methanotrophs. However, it was established that CH_4 solubility in the natural waters with a low ionic strength (<100 mM) decreases only by 1/3 at a temperature rise from 30 to 60°C [5]. This explains the occurrence of methane oxidation in different natural habitats with high temperatures.

In 1966, Foster and Davis [6] described for the first time the moderately thermophilic strain of methanotrophic bacteria, *Methylococcus capsulatus* Texas, with the optimal growth at 30–60°C. Since then, the spectrum of thermophilic and thermotolerant methanotrophs has been continuously supplemented with new taxa [7, 8]. Moderately thermophilic methanotrophs of the genera *Methylocaldum* and *Methylothermus* were isolated from hot springs in Hungary and Japan [9–12]; a thermotolerant strain Se48 belonging to the genus *Methylocystis*, was isolated from Transbaikalian hot springs [13]. Thermoacidophilic methanotrophs growing at up to 65°C were recently isolated from different geographic zones and classified within the phylum *Verrucomicrobia.* For example, *"Acidimethylosilex fumariolicum"* So IV was isolated from the mud of the Solfatara volcano in the vicinity of Naples [14]. Other thermoacidophilic methanotrophs, *"Methylokorus infernorum"* 4 and *"Methyloacida kamchatkensis"* Kam1, were isolated from a soil sample (10–15 cm, pH 4.5, 63°C) collected close to a geothermal spring in New Zealand [15] and from an acidic hot spring in Kamchatka [16], respectively.

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It was established that methanotrophs have symbiotic relations with various invertebrates (mollusks and sponges) inhabiting the areas around hydrothermal CH_4 outlets on the sea and ocean floor [17–20]. Methanotrophs, together with sulfide-oxidizing bacteria, are localized in special bacteriocyte cells of mollusk gills; the host mollusk provides the endosymbionts with the substrates from thermal spring waters (CO_2, CH_4, O_2), while bacteria, in their turn, fix carbon and supply the host with carbohydrates and amino acids.

Apart from the natural biotopes, man-made thermal ecosystems exist. Stock-farm wastes (compost, manure, and silage) are rich in organic matter and act as a kind of a natural fermentor for indigenous microflora. Intensive microbiological processes of organic matter decomposition and methane production release great amounts of heat. The calculated annual CH_4 emission from composted wastes is up to 7.4×10^6 tons and is as high as 0.31–0.41% of methane emission from the territory of Germany [21]. Moderately thermophilic representatives of the genus *Methylocaldum* were isolated from such artificial ecosystems [21, 22]. Moreover, molecular-biological techniques revealed the presence of thermotolerant and moderately thermophilic species of *Methylococcus* and *Methylocaldum* in various soils with in situ temperatures much lower than the values optimal for growth [23, 24]. Consequently, thermophilic/tolerant methanotrophs must possess high adaptability and mechanisms of survival at high and suboptimal temperatures.

TAXONOMIC DIVERSITY OF THERMOTOLERANT AND MODERATELY THERMOPHILIC METHANOTROPHS

After the description of thermotolerant strains *Mc. capsulatus* Texas and Bath [6, 25], three species of the genus *Methylococcus* were isolated and characterized, *"Mc. thermophilus", "Mc. ucrainicus"*, and *"Mc. gracile"* [26, 27]. At present, the validated taxa of moderately thermophilic and thermotolerant methanotrophs include the genera: *Methylococcus, Methylocaldum*, and *Methylothermus* (Table). They are briefly characterized below.

***Methylococcus capsulatus*.** Strains Texas and Bath have intracytoplasmic membranes (ICM) of morphotype I. They grow in the range of 30–50°C, with the optimum at 42°C. The cells are gram-negative nonmotile cocci multiplying by binary division; they are characterized by the presence of a capsule and additional layers on the outer cell wall surface (S layers) arranged in the tetragonal (*p*4) symmetry and produce dormant forms, i.e., immature cysts of *Azotobacter* type [18]. The organism utilizes only methane or methanol (0.2%) as a carbon and energy source. They possess a nitrogenase activity and can fix atmospheric nitrogen [28]. The cells of *Mc. capsulatus* contain phosphatidyl ethanolamine (74%), cardiolipin (5.8%), phosphatidyl glycerol (13%), and phosphatidyl choline, whereas phosphatidyl monomethylethanolamine and phosphatidyl dimethylethanolamine are absent. In the cellular profile of fatty acids, 16 : 1ω7c and 16 : 0 are prevalent [18, 29]. They possess both the soluble and particulate (membrane-bound) forms of methane monooxygenase (MMO). The ribulose monophosphate (RuMP) cycle is predominant, while the serine and ribulose bisphosphate (RuBP) cycles of C_1 assimilation are minor. The Krebs cycle is incomplete due to the absence of α-ketoglutarate dehydrogenase activity, but the genome contains the corresponding *kdh* gene [30]. Ammonium is assimilated via pyruvate amination with the involvement of alanine dehydrogenase and through the glutamate cycle [31–33]. These traits, together with the high DNA G + C content (60–62 mol%), which is more typical of type II methanotrophs, served as the basis for referring *Mc. capsulatus* to type "X". In view of such a "metabolic mosaic", this species is considered as an intriguing "microbial Rosetta- stone" in methanotrophy and is one of the main objects of molecular genetic studies [8].

Genus *Methylocaldum*. The methanotrophs of this genus are widespread in nature; their habitats are not only thermal springs but also activated sludge, arable lands, silage wastes, and manure [22, 21]. Representatives of the genus *Methylocaldum* (*Md. szegediense, Md. gracile*, and *Md. tepidum*) differ from *Methylococcus* in the 16S rDNA sequence (≤92% similarity) and DNA G + C content (57–58 mol%) and have no sMMO [9, 10]. *Md. gracile* and *Md. tepidum* are thermotolerant methanotrophs growing within the temperature range from 20 to 47°C, with the optimum at 42°C. Only *Md. szegediense* is a moderate thermophile, since it can grow at 62°C (optimum at 55°C) with the lower limit at 37°C.

The typical trait of the members of the genus *Methylocaldum* is cell polymorphism: their populations include, in addition to small coccoid cells, very large cocci and rods; some of them have a thick capsule resembling *Azotobacter*-type cysts; unlike typical cysts, however, they maintain a developed ICM system. Polymorphism is accompanied by variability of such other phenotypic characters as colony morphology, pigmentation, and formation of exopolysaccharides, thus complicating the determination of taxonomic position of this group of methanotrophs [10]. The instability of their growth, resulting mainly from the insufficient knowledge of their optimal physiological characteristics, often resulted in a loss of collection cultures. As can be seen from the table, the strain *Md. szegediense* O-12 has been studied most thoroughly [22].

"Methylococcus thermophilus" isolated from the bottom sediments of various water bodies, including bogs and waste treatment facilities, can grow in the range of 37–62°C, with the optimum at 56°C, pH 6.8–8.0 [26, 27]. *Mc. thermophilus* and *Methylocaldum* species were found to be phenotypically and genotypically related, because they are characterized by polymor-

Main characteristics of the thermophilic and thermotolerant methanotrophs of the phylum *Proteobacteria*

Characteristic	*Methylococcus capsulatus* [6]	*Methylocaldum gracile* [10]	*Methylocaldum tepidum* [10]	*Methylocaldum szegediense* [10, 22]	*Methylothermus thermalis* [12]	*Methylocystis* sp. Se48 [13]
Morphology	Cocci	Pleomorphic	Pleomorphic	Pleomorphic	Rounded rods	Vibrioids
Cell size (μm)	0.7–0.9 × 0.7–0.9	0.4–0.5 × 1.0–1.5	1.0–1.2 × 1.0–1.5	0.6–0.8(1.0) × × 1.0(1.8)–1.5(2.0)	0.6–0.8 × 1.0–1.2	0.6–1 × 1.2–1.7
VCM morphotype	I	I	I	I	I	II
sMMO	+	–	–	–	–	–
Motility	–	+	+	+	–	–
'Azotobacter' cysts	+	+	+	+	–	–
Colony color	White to creamy	Brownish black	Brownish black to brown	Brownish black to light-brown	Light-brown	White
t°C range (optimum)	30–55 (37–42)	20–47 (42)	30–47 (42)	37–62 (55)	37–65 (55–57)	15–53 (37)
pH range (optimum)	6.0–8.0 (6.5–7.5)	N.d.	N.d.	6–8.5 (7.2)	6.5–8.0 (6.5)	5–7.5 (5.5–6.8)
Salinity (% NaCl)	N.d.	N.d.	N.d.	0–2 (0.5)	0–3 (0.5)	0–0.5 (0)
Growth on methanol (0.1%)	+	–	–	–	+	N.d.
C_1 assimilation pathway	RuMP, RuBP, serine	RuMP, RuBP, serine	RuMP, RuBP, serine	RuMP, RuBP, serine	RuMP	Serine
α-ketoglutarate dehydrogenase	–	–	–	–	–	+
Fatty acids (% of total)						
C16 : 0	+			63.5	37.2	–
C16 : 1	+			13.3	–	–
C18 : 1	–	N.d.	N.d.	0.17	+	+
C17cyc	–			9.0	35.2	–
C9-OMe-16 : 0	–			4.6	–	–
Cardiolipin	+	N.d.	N.d.	+	–	+
Phosphatidylethanolamine	+	N.d.	N.d.	+	+	+
Phosphatidylcholine	+	N.d.	N.d.	–	–	+
Ubiquinone	Q-8	N.d.	N.d.	Methylene-Q-8	Q-8	N.d.
DNA G + C, mol%	65	56–58	57.2	56.5(58.5)	62.5	62.0
Source of isolation	Bottom deposits of water bodies, activated sludge	Activated sludge	Soil	Thermal spring Silage, manure	Thermal spring	Thermal spring

phism, formation of brown-pigmented colonies, the functioning of several C_1 assimilation pathways, and a high degree of homology of the 16S rRNA genes. The above suggests reclassification of *"Mc. thermophilus"* as *Methylocaldum szegediense*.

Genus *Methylothermus*. Strain HB isolated from the thermal springs of Hungary and Japan grew in the range of 40–70°C, with the optimum at 55–62°C, and was assigned to the novel genus *"Methylothermus"* [11]. The analysis of 16S rDNA of this strain showed its closest proximity to the genera *Methylococcus* and *Methylocaldum.* Strain HB was represented by cocci (0.6 μm) forming cream colonies. However, strain HB was soon lost and was not studied further.

The moderately thermophilic strain MYHT was isolated as a pure culture from silt samples of the Hyogo hot spring (Japan). By the 16S rDNA sequence, this strain proved to be equally close both to the partially described *"Methylothermus"* HB (91% similarity) and to the mesophilic halophile *Methylohalobius crimeensis* (90%) [34]. The isolate classified as *Methylothermus thermalis* MYHT (table) grows in the range of 37–67°C; the maximal growth rate occurs at 57–59°C [12]. Similar to *Md. szegediense*, this methanotroph has type I ICM, is salinity-dependent, grows best in the presence of 0.5% NaCl and is resistant to 3% NaCl. In contrast to *Mc. capsulatus* and *Md. szegediense*, it employs the RuMP cycle only and has no key enzymes of the serine and RuBP cycles. Moreover, PCR with specific primers did not show *Mt. thermalis* MYHT to carry the *cbbL* gene coding for the large RBPC/O subunit.

In contrast to other methanotrophs of types I or "X"characterized by predominance of hexadecanoic and hexadecenoic fatty acids, *Mt. thermalis* MYHT has equal contents of $C_{16:0}$ (37.2%) and $C_{18:1\omega9c}$ (35.2%). The presence of octadecenoic fatty acids is quite appropriate, because it is known that the membrane's melting temperature increases with extension of the length of the fatty acid carbon chain. The cellular profile of the fatty acids with the high contents of C_{16} and C_{18} fatty acids, which is unusual of type I methanotrophs, was revealed in the closest relative, i.e., the halophilic mesophile *Mh. crimeensis* optimally growing at 12% NaCl and 30°C [34]. These peculiarities of the membrane lipid composition in the new isolates call for the reassessment of significance of the fatty acidprofile as an identification marker in taxonomy and ecology of methanotrophs, particularly for their detection in extreme ecosystems.

In contrast to *Md. szegediense*, *Mt. thermalis* MYHT shows no polymorphism, which may be due to the ability to form S-layers of tetragonal (*p4*) symmetry on the cell surface; they apparently serve as an additional framework. The colonies are creamy, semitransparent, with even edges and smooth surface, 1–2 mm in diameter, become light-brown after 1-month incubation, probably due to melanin synthesis; however, this ability is poorly pronounced.

In perspective, high-temperature ecosystems are potential sources of new thermophilic methanotrophs. Extension of the spectrum of thermophilic and thermotolerant methanotrophs displays new traits of their biology and new directions of further research. Recently discovered thermoacidophilic methanotrophs are the striking example.

EXTREMELY THERMOACIDOPHILIC AEROBIC METHANOTROPHS: A NOVEL PHYLOGENETIC BRANCH OF *VERRUCOMICROBIA*

"Methylokorus infernorum". Quite recently, an obligate thermoacidophilic methanotroph ***"Methylokorus infernorum"* V4**, with the growth optimum at pH 2–2.5 and 60°C, was isolated from Hells Gates geothermal spring (New Zealand) [15]. In contrast to the known methanotrophs, this isolate is related not to the *Proteobacteria* but to the *Verrucomicrobia*, a widespread and diverse bacterial phylum consisting mainly of uncultured species with unknown genotypes.

The interesting cytological peculiarity of *Mk. infernorum* V4 is the presence of ICM as sparse tubular membrane structures attached to the inner cell membrane, similar to those in *Methylocella.* The presence of genes/enzymes of the serine and RuBP pathways, along with the absence of hexulosephosphate synthase (HPS) and tetrahydromethanopterine (THMP) dehydrogenases, suggests a novel metabolic design. The Krebs cycle is complete, but the strain does not grow on polycarbon substrates. Besides CH_4, *Mk. infernorum* V4 utilizes CH_3OH, but its growth on methanol strictly depends on CO_2. It is noteworthy that the V4 isolate has no 16 : 1ω8c, 16 : 1ω5t, or 18 : 1ω8c lipids, which are unique for methanotrophs.

"Acidimethylosilex fumariolicum". Almost simultaneously, another thermoacidophilic methanotroph was described, *"Acidimethylosilex fumariolicum"* SolV. It was isolated from the Solfatara mud volcano in the vicinity of Naples (Southern Italy) [14]. As is generally known, such volcanoes (fumaroles) are characterized by significant CH_4 emission into the atmosphere; the conditions vary from moderate temperature and neutral pH to high temperature and low pH values. In spite of the reliable indications of biological methane uptake in mud volcanoes, up to now no methanotrophic bacteria have been known that could survive under such conditions. The authors isolated strain SolV as a pure culture; based on the 16S rDNA sequence, it was classified as a member of the *Planctomycetes–Verrucomicrobia-Chlamydiae* (PVC) superphylum. This isolate can grow under CH_4 at O_2 limitation or on methanol at pH 0.8, i.e., under much more acidic conditions than the known methanotrophs.

The rod-like cells of *A. fumariolicum* SolV have a pMMO activity (50 nmol min^{-1} mg^{-1} with propylene) but do not oxidize naphthalene (negative sMMO test). The MDH activity is 60 nmol min^{-1} mg^{-1} of protein,

and the *mxa*F gene exhibited 50% similarity with *mxaF* of *Mc. capsulatus* Bath. No sMMO genes were found, but two complete *pmo*CAB operons and one *pmo*CAB with partial *pmo*C were identified. All the indicative PmoA amino acids are present, whereas the indicative amino acids of ammonium monooxygenase (AmoA) are absent. The sequences of two out of three *pmoA* genes are similar to the *pmoA* sequence obtained from the mud springs of Yellowstone National Park, which confirms the widespread occurrence of thermoacidophilic methanotrophs in extreme biotopes.

The partial analysis of genome DNA made it possible to identify many genes of C_1 metabolism, including a new combination of the serine, tetrahydrofolate (THF), and RuBP pathways of C_1 assimilation, but did not reveal the RuMP cycle genes (*hps* and *hpi*). Formaldehyde is oxidized by the THF cycle or FADH (the activity of 110 nmol min^{-1} mg^{-1} of protein).

"Methyloacida kamchatkensis". Somewhat later, a thermoacidophilic methanotroph *"Methyloacida kamchatkensis"* Kam1, isolated from an acidic hot spring near the Uzon caldera, was described [16]. The isolate also belongs to the phylum *Verrucomicrobia* and, together with other thermoacidophilic methanotrophs, forms a separate cluster. It grows in the temperature range of 37–60°C at pH 2–5 (optimal growth at 55°C, pH 3.5) in the atmosphere of $CH_4 : CO_2$: air (6 : 1 : 3) with the generation time 38 h or in the presence of low methanol concentrations (2–36 μM). It does not utilize ethanol, acetate, or glucose. Nitrogen sources are N_2, ammonium salts, and nitrates. The *pmo*A, *mmoX*, and *mxa*F genes were not revealed with the standard primers; therefore, atypical genes/enzymes of methane and methanol oxidation are probably functioning. Instead of ICM stacks, the strain forms polyhedral intracellular organelles resembling carboxysomes of cyanobacteria and chemoautotrophs. The functionality of these organelles is still unknown, but it is possible that they serve for compartmentation of an unusual MMO or RuBPC/O.

Three new isolates of thermoacidophilic methanotrophs are phylogenetically close and, most likely, are different species of the same genus, for which the name of *Methylacidiphilum* was proposed.

These sensational discoveries demonstrated that methanotrophy in bacteria is more diverse in the taxonomic, ecophysiological and genetic aspects than it has been considered earlier and that the previous studies did not provide complete insight into the diversity of methanotrophs in thermal and acidic biotopes. Further detailed studies are needed for decoding the mechanisms of survival and evolution of these new representatives of methanotrophic bacteria, which truly surmount all barriers and overpass all boundaries.

TRAITS OF C_1 METABOLISM IN *Methylococcus capsulatus* BATH

Methanotrophs obtain the energy and reducing equivalents necessary for the biosynthesis of triose phosphates by sequential oxidation of methane to CO_2 via a series of enzymatic transformations, with methanol, formaldehyde, and formate as intermediates. The main properties of the enzymes of the primary C_1 metabolism, exemplified by *Mc. capsulatus* Bath, will be briefly considered below.

Mc. capsulatus Bath has both soluble and particulate MMOs; their expression is not coupled and is regulated at the genetic level by Cu^{2+} concentrations in the medium [35–37].

Soluble methane monooxygenase (sMMO) of *Mc. capsulatus* Bath has three components (hydroxylase, reductase, and regulatory protein B), which are encoded by the genes located in the *mmoXYBZDC* operon. Hydroxylase is an oligomer (250 kDa) consisting of three subunits: α_2-binuclear nonheme iron center (60 kDa), β_2 (45 kDa), and γ_2 (20 kDa) encoded by the *mmoX*, *mmoY* and *mmoZ* genes, respectively [38]. Reductase (38.4 kDa) comprises FAD and a (Fe_2S_2) cluster and is encoded by the *mmoC* gene. The reductase accepts electrons from NADH and transfers them to the diferric site of the hydroxylase. Regulatory protein B (15.8 kDa) encoded by the *mmo*B gene has no prosthetic group and cofactor but binds to the hydroxylase and is necessary for the sMMO activity [39, 40]. The function of the mmoD protein is still unknown, though it is supposed to play some role in the assembly of the active site of the reductase [41].

A broad range of aliphatic, aromatic, heterocyclic, acyclic, and halogenated hydrocarbons are cooxidized by sMMO [42]. However, sMMO functions only at low copper concentrations in the medium (<0.08 μM); therefore, it is difficult to use this enzyme for bioremediation since contaminated ecosystems with low copper content are rather rare.

Particulate methane monooxygenase (pMMO). The presence of Cu^{2+} ions in the growth medium results in the expression of pMMO, which amounts to 60–80% of the total membranous proteins in the cell [43]. The pMMO genes are arranged into the *pmo*CAB operon and code for the polypeptides α, β and γ with supposed molecular masses of 46, 28 and 29 kDa, respectively [44]. *Mc. capsulatus* Bath possesses two nearly identical copies of this operon and the third copy of the *pmo*C gene [45], the nucleotide sequence of which differs from the two analogous genes of the operons. Mutations in these genes revealed the necessity of both operons for the maximal pMMO activity.

Although pMMO is an extremely unstable enzyme, it was found to be a heterotrimer with the molecular mass of ~100 kDa [46]. Gel electrophoresis in the presence of nondenaturing agents (perfluorooctanic acid, etc.) demonstrated the molecular mass of pMMO to be 200 kDa, which corresponds to a hexamer $(\alpha\beta\gamma)_2$. Other

variants are possible as well, because native gel electrophoresis revealed an additional protein band with the molecular mass of ~440 kDa, which may correspond to a tetramer [47].

The active center of pMMO contains two iron atoms and approximately 15 copper atoms per enzyme molecule; each of them seems to be involved in the catalysis. For expression and functioning, pMMO requires a small fluorescent chromopeptide, methanobactin (1.2 kDa), which binds exogenous Cu atoms and transports them to pMMO [48–50]. The electrons from pMMO are transferred to cytochromes $b_{559/569}$ or c_{553}. It is notable that pMMO has high affinity to methane (K_m = 1–2 μM) and oxygen (K_m = 0.1 μM) but narrow substrate specificity, co-oxidizing only short alkanes, alkenes, and methanol [38, 51, 52].

Recently, Dalton and coworkers demonstrated the mutual position of pMMO and MDH in the membranes of *Mc. capsulatus* Bath. The analysis of individual particles with 16 Å resolution using cryoelectron microscopy allowed the authors to report for the first time the three-dimensional structure of the super-complex, oxidizing methane to formaldehyde [53]. An original model was proposed, where pMMO-H looks as a cylinder body and the MDH domain looks like a "cap". The postulated mechanism for the assembly of pMMO and MDH into a complex determines their stability and facilitates direct electron transfer between them, providing more efficient (35%) methane utilization [54].

Methanol dehydrogenase catalyzes methanol oxidation to formaldehyde. It is a heterotetramer $\alpha_2\beta_2$, consisting of two α (67 kDa) and two β (8.5 kDa) subunits encoded by the *mxaF* and *mxaI* genes, respectively [35, 55]. Two α-subunits coupled by a characteristic motif form a "propeller"-shaped structure [56]. PQQ molecules and Ca^{2+} ions are localized in a funnel-shaped central channel. The β-subunits do not form functional MDH domains, and the function of this protein has not yet been elucidated [57].

Formaldehyde and formate dehydrogenases are involved in the pathway of direct formaldehyde oxidation to CO_2. The major pool of NADH for MMO is formed at formaldehyde oxidation via formate to CO_2. Methanotrophs possess several enzymes of formaldehyde oxidation. These enzymes belong to two groups: $NAD(P)^+$-dependent and cytochrome-bound. In turn, $NAD(P)^+$-dependent dehydrogenases are divided into three subgroups depending on the secondary cofactors: reduced glutathione (GSH), tetrahydrofolates (THF, H_4F), or tetrahydromethanopterins (H_4MPT/THMP). The sequences of the genes coding for the enzymes of the THF and THMP pathways were found in the genome of *Mc. capsulatus* Bath [30]. The THF pathway is used for formaldehyde and formate assimilation in the serine cycle, whereas the THMP pathway is used mainly for formaldehyde oxidation to formate by highly active dehydrogenases similar to those in *M. extorquens* AM1 [58].

Hexulose-phosphate synthase (HPS) catalyzes the key reaction of the RuMP pathway: condensation of formaldehyde and ribulose-5-phosphate with the formation of a thermolabile 3-hexulose-6-phosphate; the latter is isomerized into fructose-6-phosphate by phosphohexuloisomerase (PHI). HPS of *Mc. capsulatus* Bath is a hexameric (310 kDa) membrane-bound enzyme. On the contrary, HPS in methylobacteria is a soluble dimeric or monomeric protein of 15.5–45 kDa [59]. The genome of *Mc. capsulatus* Bath was found to carry two identical direct repeats, 5267 bp, separated by the transaldolase gene, containing the copies of the *hps* and *pgi* genes coding for HPS and PHI, respectively [30]. According to the genomic data, the molecular mass of HPS corresponds to the product of fusion of the *hps* and *pgi* genes. Interestingly, the hyperthermophile *Pyrococcus horikoshi* was found to carry the gene coding for the membrane-bound HPS–PHI hybrid protein. The fusion of two enzymes with the formation of a hybrid apparently enhances the efficiency of the isomerase reaction, because the product of the former reaction, 3-hexulose-6-phosphate, is extremely unstable at high temperatures [60].

One more interesting feature of the constructive metabolism of *Mc. capsulatus* Bath is the presence of **pyrophosphate (PPi)-dependent 6-phosphofructokinase** (PFK), which paradoxically differs in the kinetic properties and amino acid sequence from the analogous enzyme of *Mm. methanica* 12 (16% identity) but is close enough to other homologues (70–75%) from autotrophic prokaryotes. Possessing the highest affinity to ribulose-5P and particularly to sedoheptulose-7P rather than to fructose-6P, PFK probably participates in regeneration of ribulose-1,5P_2, which is the primary acceptor of CO_2, in the minor Calvin cycle. It was also revealed that the genes of PFK (*pfp*) and the membrane H^+-translocating pyrophosphatase (*hpp*) were co-transcribed in the single bicistronic operon, indicating possible involvement of the tandem of these enzymes in the energy metabolism of *Mc. capsulatus* Bath. Confirmation of the conceptual justification of these original hypotheses needs additional experimental evidence [61, 62].

Being a peculiar "roadmap", complete genome mapping of *Mc. capsulatus* Bath has made it possible for the first time to present an integral picture of the metabolic organization of this "mosaic" methanotroph. For example, the newly found protein hemerythrin (McHr) is involved in oxygen transport and expressed at a high Cu to biomass ratio. An iron-containing domain localized in the hydrophobic pocket was identified within McHr; it participates in the O_2 binding. The exact function of hemerythrin is unknown but, since it is expressed at a high Cu concentration in the medium, the involvement of McHr in methane oxidation is not excluded [63].

GENOMIC PREDICTIONS AND METABOLIC RECONSTRUCTIONS OF *"Methylacidiphilum infernorum"*

Genome analysis made it possible to reconstruct the pathways of the primary and central metabolism in *"Ma. infernorum"*. In spite of the small genome size (2.3 Mbp), this methanotroph possesses the necessary set of enzymes for methylotrophic growth but lacks many enzymes of the carbohydrate metabolism. Along with the presence of three complete *pmoCAB* operons coding for the particulate MMO, with two of them coupled, another locus of DNA has an additional (the fourth) *pmoC* gene, thus implying some other role of this subunit [64]. Three MMOs probably have different sensitivity to competitive inhibition by ammonium or different kinetic properties, temperature, and pH optima. The sMMO genes have not been revealed. The product of CH_4 oxidation, methanol, seems to be oxidized by an MDH homologue. However, the genome of *"Ma. infernorum"* comprises the *mxaF* gene coding for the large subunit of the enzyme but not the *mxaI* gene coding for the small subunit of MDH needed for activity in *Mc. capsulatus* Bath and in a number of methylobacteria. It is probable that a methanol-binding periplasmic protein and cytochrome *c*, the genes of which are coupled with *mxaF*, are involved in the oxidation. Interestingly, there is a whole cluster of genes coding for the enzymes of the PQQ cofactor (*pqqABCDE*) biosynthesis. The known genetic modules of formaldehyde oxidation via the THMP pathway, methylene-THF dehydrogenase, and methenyl-THF cyclohydrolase, which are typical of methylotrophic bacteria, are absent. Like many other bacteria, *"Ma. infernorum"* employs the *folD* gene product for formaldehyde oxidation. It implies the operation of new methylotrophic pathways and the ancient divergence of methanotrophs of the *Verrucomicrobia* and *Proteobacteria* rather than simple horizontal transfer of the methanotrophy genes [65].

Formaldehyde is assimilated through the RuBP and serine pathways. The RuMP cycle does not participate in C_1 assimilation due to the absence of respective HPS and HPI genes (*hps/hpi*) and distal enzymes of the Entner–Doudoroff pathway: 6-phosphogluconate dehydratase (Kdd) and 2-keto-3-deoxy-6-phosphogluconate aldolase (Eda).

As regards the serine pathway, the absence of the two key genes/enzymes, glycerate kinase and malyl-CoA lyase, suggests the operation of alternative variants of glyoxylate regeneration. One of them is the reaction of ribulose-1,5-bisphosphate oxygenation with the formation of phosphoglycolate, catalyzed by RuBPC/O, which is followed by conversion of phosphoglycolate into glyoxylate. The reactions of the glyoxylate bypass are one more source of glyoxylate for the functioning of the serine pathway in this methanotroph. The cycle of glyoxylate regeneration through polyhydroxybutyrate formation proposed for *M. extorquens* AM1 [66] does not operate in *"Ma. infernorum"*. There is an indication of direct coupling of the serine and pentose-phosphate pathways through an unusual protein possessing the serine transhydroxymethylase domain and two domains homologous to phosphatase and phosphoriboisomerase B.

Like other thermophiles, *"Ma. infernorum"* has an anti-stress system containing a polymerase cassette, which is a determinant of thermophily. Besides, one cannot rule out the involvement of a novel antiviral system; its components were found along with the classical restriction–modification system. The additional DNA locus encoding DNA methylases, helicases, and nucleases may play an analogous protective role [64].

These and other hypothetical metabolomic reconstructions and extrapolations based on genome analysis, however interesting, are not yet confirmed by appropriate enzymological data. The reasoning of the authors of the annotated genome as regards the origin and evolution of metabolic pathways in *"Ma. infernorum,"*, while attractive in the conceptual aspect, is not quite convincing. For instance, it is stated that in the course of its existence the organism has lost two times as many genes (526) than it has received (262) from *Proteobacteria*.

TEMPERATURE-INDUCED CYTOBIOCHEMICAL REARRANGEMENTS IN THERMOPHILIC AND THERMOTOLERANT METHANOTROPHS

The causes of polymorphism typical of methanotrophs of the genus *Methylocaldum* were studied on *Md. szegediense* O-12. After the culture grown at the optimal temperature (57°C) was transferred to a lower temperature (37°C), the cells of this methanotroph became irregularly shaped and much larger. After 5–7 days of adaptation to 37°C, the culture contained mainly small coccoids that lost their growth ability after several transfers. Staining of the cells grown at 37°C with DAPI, a fluorochrome selectively binding to DNA, revealed numerous nucleoids in the giant cells. Polymorphism and death of cells were therefore supposed to result from impaired cell division under stress impacts, first of all, temperature changes. Indeed, under routine cultivation and maintenance of the cultures of thermophilic methanotrophs, which requires periodical replacement of the gas phase, the temperature shift may exceed 30°C. However, during the cultivation of *Md. szegediense* O-12 at 55°C with all necessary precautions taken against thermal stress (introduction of warm inoculum into the medium preheated to the cultivation temperature and maintenance of stable temperature conditions at gas phase replacement), cell polymorphism was less marked [22]. Determination of adequate conditions of growth and maintenance is therefore the necessary prerequisite for the work with thermophilic methanotrophs.

The ability of *Md. szegediense* O-12 to restore membrane fluidity to the optimal level after temperature changes is evidence of the functioning of thermoadaptive mechanisms typical of prokaryotes. For example, in the cells grown at 55°C, saturated fatty acids ($C_{16:0}$) were predominant and methylated, and cyclic derivatives of fatty acids ($C_{16:0\text{-OMe-9}}$ and C_{17cyc}) atypical of eubacteria were present in considerable amounts. At the cultivation temperature decreased to 42 or 37°C, the content of unsaturated fatty acids increased but their methylated derivatives were absent. Saturated fatty acids are known to participate in stabilization of the cell membrane structure by reducing its fluidity at high temperatures, whereas methylated and cyclic fatty acids restrict ion transport into the cells [67].

Besides the unusual isomers of fatty acids, the membranes of *Mc. capsulatus* Bath were shown to contain sterols (squalene) [68–70]. The biosynthesis of these compounds is infrequent in prokaryotes. It is believed that sterols give the membranes a more rigid structure, providing the adaptation of *Mc. capsulatus* Bath to environmental conditions. Other specific functions of sterols are probable as well.

Due to the mapping of the *Mc. capsulatus* Bath genome, it was possible to identify the pathway of sterol biosynthesis. At first, 6 molecules of acetyl-CoA are condensed with the formation of squalene. Then squalene monooxygenase catalyses epoxidation of squalene into (3S)-2,3-oxidosqualene, which is cycled with the formation of lanosterol, followed by C-32 removal by cytochrome P_{450}-14-demethylase. Squalene monooxygenase is a membrane-bound enzyme of 45 kDa, while oxidosqualene cyclase is a cytoplasmic protein of 72 kDa [70]. On the contrary, cytochrome P_{450}-14-demethylase catalyzing the third stage of the sterol biosynthesis pathway is a soluble protein of 62 kDa. The gene of cytochrome P_{450}-14-demethylase transcribed backwards (in the opposite direction) relative to the genes of squalene monooxygenase and oxidosqualene cyclase, also participates in the synthesis of hopanoids. Hopanoids are widespread among prokaryotes and involved in membrane stabilization. The unique triterpenes of the hopanoid series were found in the representatives of *Methylocaldum.* Triterpenes of the hopanoid series, aminopentol with the Δ^{11} double bond, were found in *Mc. capsulatus, M. methanica, Mc. luteus, Md. szegediense*, and *Md. tepidum* [71].

Bacteria of the genus *Methylocaldum* and *Mc. capsulatus* belong to the "X" type methanotrophs; they are characterized by simultaneous operation of three C_1 assimilation pathways: the RuMP cycle, the serine pathway, and the Calvin cycle. The levels of the key enzymes of these pathways in *Md. szegediense* depended on growth temperature; while the activity of HPS, the key enzyme of the RuMP cycle remained permanently high, the level of RuBPC/O, the specific enzyme of the Calvin cycle, increased twofold and the activity of hydroxypyruvate reductase (HPR), the indicator enzyme of the serine pathway, increased by an order of magnitude in the cells grown at 55°C as compared to those growing at 37°C. The role of these metabolic rearrangements is obscure, although activation of the enzymes of the serine and RuBP cycles, more energy-consuming than the RuMP cycle, in response to the growth temperature increase, points to intensification of the carbon flux through energy-consuming pathways. This probably provides dissipation of the excess of energy and protects the cells from overheating.

In addition to the mentioned cytobiochemical traits, some enzymes of C_1 metabolism in *Md. szegediense* O-12 possess specific properties. At least FDH, HPR, RBPC/O, and HPS are most active at higher measurement temperatures (50–65°C) as compared with the same enzymes of *Mc. capsulatus* Bath (40–50°C). Probably, this is one more evolutionally retained adaptation of methanotrophs to high temperatures typical of the habitats of thermophilic microorganisms [72].

The biosynthesis and role of sucrose in thermoadaptation of methanotrophs. *Md. szegediense* O-12 was shown to contain sucrose, and its content increased at higher salinity of the medium and cultivation temperature, reaching maximum values (1.2%) in the cells grown at 57°C with 1% NaCl. On the contrary, *Mc. capsulatus* Bath did not accumulate sucrose in all of the tested cultivation modes (37–45°C) [72].

The capacity for sucrose accumulation correlates with the higher halotolerance of *Md. szegediense* O-12, which indirectly indicates the role of this disaccharide as an osmoprotectant. It is, however, quite possible that sucrose synthesis is associated primarily with thermoadaptation. Increased turgor of the cytoplasm may be among the consequences of sucrose accumulation, making it necessary to equalize the internal osmotic pressure by enhancement of ionic strength in the medium and explaining the stimulation of culture growth by NaCl addition. Moderate halotolerance is also typical of *Mt. thermalis* MYTH; therefore, it should be expected that among thermophilic methanotrophs, sucrose synthesis is a rather widespread mechanism for stabilization of biomolecules and cell structures.

Md. szegediense O-12 was shown to possess the activities of sucrose phosphate synthase, condensing UDP-glucose and fructose-6-phosphate, and sucrose phosphate phosphatase, dephosphorylating sucrose-6-phosphate with sucrose formation. This methanotroph therefore synthesizes sucrose from the primary intermediates of the RuMP cycle [72]. It is notable that the activity of sucrose phosphate synthase is higher in the cells grown at 55°C than in the cells grown at 42°C but is not modulated by NaCl. This fact confirms the assumption that sucrose accumulation in *Md. szegediense* O-12 is associated with thermoadaptation rather than with osmoadaptation. The presence of the sucrose phosphorylase activity (degrading sucrose to glucose-1-phosphate and fructose) indicates the possibility of

its reutilization, considering the presence of hexokinase. Accordingly, sucrose may be considered not only as a thermoprotectant but also as a reserve carbon substrate increasing the survival ability of this methanotroph under methane deficiency.

The thermostabilizing effect of sucrose was demonstrated in the experiments with cell-free extracts of *Md. szegediense* O-12 and *Mc. capsulatus* Bath or a preparation of lactate dehydrogenase, which were exposed to 50 or 60°C prior to determination of enzyme activities at 30°C. Residual enzyme activities were higher in case of preheating in the presence of sucrose.

In addition to the thermoprotective effect, the energy-consuming sucrose synthesis may serve as a regulatory mechanism for the cell energy state. It is not improbable that thermophilic methanotrophs synthesize, besides sucrose, some other thermoprotectants, which have not yet been identified. Identification and quantitative assessment of protective compounds, genes, and enzymes of their biosynthesis in methanotrophs will promote the detection of new biochemical pathways and expansion of the biotechnological potential of these bacteria.

Sucrose is one of the main biosynthetic products, namely, the key carbohydrate of plants [73]. Sucrose and the enzymes of its synthesis were found in cyanobacteria and in several proteobacterial species which have the Calvin cycle. Among methanotrophs, only thermo- and halophilic species with the RuMP cycle accumulate this carbohydrate, whereas thermotolerant *Mc. capsulatus* Bath does not accumulate sucrose and has no respective genes and biosynthetic enzymes. This is an indication of the protective function of sucrose at stress impacts, apparently analogous to that shown for trehalose, and is in agreement with the experiments where the survival rate of *E. coli* cells increased many times after introduction of the gene of sucrose phosphate synthase [74, 75]. Consequently, the methanotrophs from extreme ecosystems have acquired the ability to synthesize sucrose in the course of evolution [72, 76, 77].

The synthesis and role of the melanin pigment in *Methylocaldum szegediense*. Melanins are negatively charged hydrophobic high-molecular pigments formed by oxidative polymerization of aromatic (phenolic or indole) substances. The synthesis of these pigments was revealed in a number of pro- and eukaryotes, and association of this process with protection from various stress factors was shown. Among the known methanotrophs, only representatives of the genus *Methylocaldum* (*Md. szegediense, Md. gracilis*, and *Md. tepidum*) produce a melanin-like pigment [10, 26, 27]. Dark-brown pigmentation was also found in the mesophilic methanotroph *Methylosinus sporium*; however, it results from formation of a stained siderophore involved in transport of Fe ions at their deficiency in the culture medium [78].

The nature, mechanism of synthesis, and role of melanin have been studied in *Md. szegediense* O-12 [79]. The production of a brown pigment in *Md. szegediense* O-12 was induced by the lowering of the cultivation temperature, because the colonies were usually cream-colored at the optimal growth temperature and gradually became dark-brown at a lower temperature (45°C), but the pigment did not diffuse into agarized medium. Pigmentation was absent at a slower growth of the strain at 37°C. The EPR spectra of *Md. szegediense* O-12 cells grown at 45°C and of the pigment isolated from the latter demonstrated the presence of free radicals in its composition and corresponded to the spectra of microbial melanins.

The culture liquid of *Md. szegediense* O-12 grown at 45°C accumulated up to eleven aromatic substances; two of them were identified as 4-hydroxyphenylacetic and 4-hydroxyphenylpropionic acids. The accumulation of these compounds is evidence of disturbances in the pathways of tyrosine catabolism. Indeed, the activity of aminotransferase, which transfers the amino group of tyrosine to α-ketoglutarate (with the formation of glutamate and 4-hydroxyphenyl pyruvate), and the activity of tyrosine decarboxylase (with the formation of tyramine) were higher in the cells grown at 45°C than in those grown at 55°C. In contrast, the subsequent reactions of degradation of the intermediates through homogentisic acid with the involvement of homogentisate-1,2-dioxygenase and maleylacetoacetate isomerase [80, 81] either declined or were not observed in the cells grown at suboptimal temperature. Consequently, melanization of *Md. szegediense* O-12 results from accumulation of the intermediates of incomplete tyrosine degradation, primarily homogentisic acid, due to the decrease of the growth temperature, followed by their spontaneous oxidative polymerization. *Mc. capsulatus* Bath executes a different way of regulation of the level of aromatic amino acids, because the activities of homogentisate-1,2-dioxygenase and maleylacetoacetate isomerase were not detected.

It is known that melanin in some microorganisms performs a protective function, protecting the cells from temperature changes and reactive oxygen species (ROS). The analogous function of melanization may be suggested for methanotrophs. On the one hand, it is an alternative way of removal of aromatic toxicants, probably involving ROS. On the other hand, melanin containing numerous double bonds can be considered as a "trap" for ROS. Melanization is therefore, a device for protection of methanotrophs from oxidative stress, apparently associated with the instability of the pMMO complex functioning at temperature fluctuations. Accordingly, it would be interesting to elucidate the mechanism of "overproduction" of the melanin precursor, tyrosine. One of the possible reasons for accumulation of this amino acid is the lower rate of protein synthesis at lower temperatures. Methanotrophs of the genus *Methylocaldum* are potential model organisms for studying the role and regulation of melanin biosyn-

thesis. For interpretation of the true role of melanin, it is necessary to obtain the mutants unable to synthesize this pigment and to study their sensitivity/resistance to the action of physical factors (temperature, pH, salinity).

Antioxidant systems. One of the consequences of the effect of high temperature on microorganisms is a higher level of ROS including H_2O_2, hydroxyl radical $OH^{\bullet}$, and superoxide anion $^{\bullet}O_2^-$, which suggests that this type of stress is oxidative [82]. ROS levels in adapted cells are maintained within the physiological range due to the functioning of specialized protective systems: catalase, superoxide dismutase (SOD), cytochrome *c* peroxidase, glutathione peroxidase, and free glutathione. The increase of these enzyme activities is an indicator of oxidative stress.

Methanotrophs *Md. szegediense* O-12 and *Mc. capsulatus* Bath growing at optimal temperatures were shown to produce ROS more intensively than at suboptimal temperatures and have higher cytochrome *c* peroxidase activities. Peroxidase activity is associated with the membranes and is more intensive in the cells grown at high temperature. In *Md. szegediense* O-12, peroxidase activity is determined by a heme-containing protein (80 kDa). Obviously, enhanced rates of ROS production result from the high intensity of the metabolic processes of methane oxidation and assimilation, because at least CH_4 oxygenation (involving pMMO and oxygenase activity of RuBPC/O) leads to the formation of hydrogen peroxide and other ROS. On the contrary, the levels of glutathione, glutathione peroxidase, and glucose-6-phosphate dehydrogenase in *Md. szegediense* O-12 increased in response to a drop in growth temperature. At the same time, an increased level of peroxidate lipids was revealed by measuring the interaction between cell components and thiobarbituric acid. In this strain, lower cultivation temperatures probably impair the membrane structure and, as a consequence, the coupling of membrane-related reactions, which induces the formation of peroxide forms of fatty acids. The process of their utilization involves mainly glutathione and glutathione peroxidase, as well as glucose-6-phosphate dehydrogenase that supplies NADPH for GSSG reduction by glutathione reductase. In contrast to *Md. szegediense* O-12, the degree of lipid degradation and the intracellular level of glutathione in *Mc. capsulatus* Bath are higher at cultivation temperatures close to the optimum (45°C), showing different functions of this tripeptide in thermoadaptation of methanotrophs.

Fifty-seven proteins bearing the heme-binding motif were identified in the genome of *Mc. capsulatus* Bath; five of them are members of the family of cytochromes c_{553} and four have been tentatively annotated as cytochrome *c* peroxidases [30, 83–85]. Cytochrome *c* peroxidase isolated from *Mc. capsulatus* Bath is a homodimer with 35.8-kDa subunits, localized in the periplasm. This enzyme is supposed to participate in H_2O_2 detoxification and methylamine oxidation [86]. Besides, the genome of *Mc. capsulatus* Bath was shown to contain an open reading frame (ORF) coding for the diheme cytochrome *c* peroxidase MCA2590, which also uses the heme as a cofactor and reduces H_2O_2 to H_2O [87]. Since the *mca*2590 gene is under the same promoter as *mop*E, the expression of which is suppressed by copper ions, the level of MCA2590 expression is also regulated by Cu content in the medium. *In silico* analysis of MCA2590 demonstrated that the protein after processing (cleavage between Ala_{41} and His_{42}) consists of 732 amino acids and has the molecular mass of 78 kDa. Protein MCA2590 has two regions with high similarity to *c*-type cytochromes and the cytochrome-binding motifs CX_2CH. Alignment of the amino acid sequence of protein MCA2590 showed a certain similarity to the well-described cytochrome *c* peroxidase from *Nitrosomonas europaea* and methylamine-utilizing protein MauG from *Paracoccus denitrificans*. In spite of the fact that the similarity to these proteins is below 30%, their differences from MCA2590 practically do not affect the functional area; hence, MCA2590 and analogous proteins can be referred to a novel group of bacterial peroxidases differing from diheme cytochrome *c* peroxidases in additional elements of the secondary structure. The immune assay and biotinylation showed the localization of protein MCA2590 on the cell surface of *Mc. capsulatus* Bath, in contrast to the periplasmic localization of the known diheme cytochrome *c* peroxidases. Although they perform a protective function in the periplasm, thus reducing the peroxides formed in the oxidative metabolism, the greater size and extracellular localization of MCA2590 imply other physiological functions [87]. A sequence (840 bp) with 43% homology to the surface protein Mca 2590 of *Md. szegediense* Bath was identified in the DNA of *Md. szegediense* O-12.

The genome of *Mc. capsulatus* Bath also contains a gene encoding the GSH-peroxidase [30]. The catalase activity was found in neither of the methanotrophs; besides, the genome of *Mc. capsulatus* Bath lacked the genes coding for this enzyme. The absence of catalase in methanotrophs is quite understandable due to involvement of hydrogen peroxide in the reaction of methane oxidation by methane monooxygenase. At the same time, the SOD activity independent of cultivation temperature was revealed.

Thus, the ROS protection systems of *Md. szegediense* O-12 and *Mc. capsulatus* Bath at the optimal and suboptimal cultivation temperatures are different. At higher (close to optimum) growth temperatures, the cells of both methanotrophs express cytochrome *c* peroxidase against the background of unchanged SOD activity and the absence of catalase; however, *Md. szegediense* O-12 additionally accumulates free glutathione in the cells and expresses glutathione peroxidase in response to a decrease in growth temperature. Bacteria of the genus *Methylocaldum* are found not only in high-temperature ecosystems, i.e., thermal

springs and wastes of different heat-producing facilities, but also in the biotopes where the temperature is subject to considerable fluctuations: stock-farm wastes, rice fields, etc. It is probable that the traits of the oxidant protection systems of *Md. szegediense* O-12 are associated with the higher degree of its thermophily and the need of adaptation to greater temperature shifts.

SURFACE PROTEINS OF *METHYLOCOCCUS CAPSULATUS* BATH

Membrane-bound proteins MopA, MopB, MopC, MopD, and MopE with the conjectural molecular masses of 27, 40, 46, 59, and 66 kDa, respectively, were found on the cell surface of *Mc. capsulatus* Bath [88]. At the same time, MopC and MopD exist in vivo as a heterodimer (95 kDa), forming a porin that probably participates in the transport of copper ions. In the C-terminal region of protein MopB, the conservative domain was revealed, which is typical of outer cellular proteins of the OmpA family and involved in the transport of substrates as well as in maintenance of the cell structure, while specific transmembrane domains are localized in the N-terminal region [89].

Hybridization of the antibodies to protein MopE confirmed its presence in the cells of *Mc. capsulatus* Bath and the presence of a 45-kDa protein in the culture liquid [90]. Subsequent sequencing of the *mop*E gene and comparison with the N-terminal sequence of the 45-kDa protein showed that it is a C-terminal peptide of the protein MopE, where the peptide is cleaved after Ala_{204}. The quantity of the excreted peptide was rather high and reached about 0.4% of the biomass weight. Comparison with the known proteins revealed similarity (in the C-terminal region only) to the protein CorA of the methanotroph *Methylomicrobium album* BG8, the function of which is supposedly the transport and accumulation of copper ions. It was shown that mature MopE designated as $MopE^C$ and the 45-kDa peptide (MopE*) excreted into the medium are synthesized in response to the absence or low content of Cu ions in the cultivation medium. However, in contrast to the known copper-binding proteins, the MopE sequence does not contain the typical region of Cu binding: methionine-X-cysteine-X-X-cysteine [91].

Taking into consideration the key role of copper in the metabolism of methanotrophs, it was supposed that they must possess specific compounds involved in the binding, transport, accumulation, and detoxification of Cu ions. Originally, a copper-binding compound associated with pMMO was found in *Mc. capsulatus* Bath [92]. Further studies confirmed the presence in methanotrophs of methanobactin (Mb) analogous to Fe-siderophores: pyoverdine and azotobactin [49, 93]. According to the data of X-ray structure analysis, this chromopeptide $C_{45}N_{12}O_{14}H_{62}Cu$ (1.2 kDa) is shaped as a compact pyramid based on a chromophore, 4-thionyl-5-hydroxy imidazole binding Cu ions in the stoichiometric ratio of 1:1.

The purified Mb preserved the biological activity and stimulated growth by reducing the lag phase of the culture at a "shock" by increased concentration of Cu ions [49, 94]. Addition of the Mb–Cu complex to the washed membrane's fraction of *Mc. capsulatus* Bath enhanced the pMMO activity by 50–75%, because this complex increased the electron flow to pMMO [93]. Moreover, quite recently the involvement of Mb in dismutation of the superoxide anion $^{\bullet}O_2^-$ into H_2O_2 and reduction of H_2O_2 to H_2O without the formation of hydroxyl radical $OH^{\bullet}$ was reported [95]. Although the physiological role and chemical structure of Mb are known, the molecular mechanisms of its transport and interaction with pMMO are still to be interpreted. Unlike the structurally and functionally similar Fe-siderophores, Mb can bind a wide range of heavy metals including radionuclides. This feature of Mb suggests an important ecological role of thermophilic methanotrophs in solubilization/mobilization and removal of many metals and radionuclides, which can be applied, in particular, for bioremediation of water used for the cooling of reactors of nuclear power plants or uranium mine dumps.

In conclusion, let us note that thermophiles are an important microbial resource for potential application in scientific research and practice. About 100 genera and 200 species of hyperthermophiles are known; among them, more than 20 genomes and proteomes have been sequenced. Further work in this promising direction will contribute to the search and identification of novel thermostable genes and enzymes and new metabolic pathways. Thermostable enzymes (thermozymes) are used in molecular biology for research and diagnostics (DNA/RNA processing) and in different fields of biotechnology (starch conversion, food industry, treatment of industrial discharge, organic synthesis, paper, and tanning industries).

Our studies on the mechanisms of thermoadaptation of methanotrophs revealed the cytobiochemical traits which are of interest for biotechnology. It was shown that some enzymes of the C_1 metabolism have optimum activity in the range of 55–70°C; hence, these thermozymes can be obtained and applied for bioremediation of various pollutants, biosynthesis of methanol and other target products from relatively cheap and renewable substrates, i.e. methane. In particular, the ability of methanotrophs of the genus *Methylocaldum* to synthesize melanin can be used for obtaining the bioprotectants applied in medicine against the action of mutagenic and carcinogenic factors.

In general, it should be acknowledged that our understanding of biology of thermophilic methanotrophs is superficial and fragmentary. In spite of the impetuous progress in our knowledge, many aspects of the structural and functional thermoadaptation of methanotrophs have not been interpreted and are descriptive. The search for new thermophilic/tolerant methanotrophs implementing unusual strategies and mechanisms

of survival at high temperatures is of evident scientific and practical interest. New horizons are opened up on the thorny but fascinating path to the knowledge of the origin and evolution of methanotrophic genomes, proteomes, and metabolomes. The actively developed unique technology of microcultivation in miniature (1 μl) bioreactors, using nanoelectron sensors and biochips, allows the monitoring of adequate stress responses of single cells and promises interesting opportunities in this respect [96].

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