Physiology and Biochemistry of the Aerobic Methanotrophs

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Abstract

Methanotrophs are a widely distributed group of aerobic bacteria that use methane as their source of carbon and energy. They play key roles in the global carbon cycle, including controlling anthropogenic and natural emissions of the greenhouse gas methane. Methanotrophs oxidize methane using the unique enzyme methane monooxygenase which exists in two structurally and biochemically distinct forms. One form, the membrane-associated or particulate methane monooxygenase (pMMO), is found in most known methanotrophs and is located in the cytoplasmic membrane. Another form, the soluble methane monooxygenase (sMMO), is found in some methanotrophs and is located in the cytoplasm. Both forms of MMO can co-oxidize a range of hydrocarbons and chlorinated pollutants and hence are interesting with respect to the biotechnological potential of methanotrophs. Methanol is further oxidized to formaldehyde, formate, and CO_2, by specific methylotrophic enzymes, while biomass is built from formaldehyde, formate, CO_2, or a combination thereof via three cyclic biochemical pathways: the ribulose monophosphate (RuMP) cycle, the serine pathway, and the Calvin-Benson-Bassham (CBB) cycle. The availability of genome sequences of methanotrophs enables postgenomic studies to investigate the regulation of methane oxidation in the laboratory and in the environment by natural methanotrophs and in laboratory or industrial conditions by platform organisms. Recent studies have included synthetic biology approaches and in future may incorporate the design of new pathways.

1 Introduction

Methane-oxidizing bacteria (methanotrophs) are a widely distributed group of aerobic microorganisms that use methane as their source of carbon and energy. Methanotrophic bacteria belong to the phyla *Proteobacteria* (Hanson and Hanson 1996) and *Verrucomicrobia*. They include extremely acidophilic methanotrophs (Op den Camp et al. 2009) and the candidate division NC10. NC10 includes a candidate oxygenic methanotroph that oxidizes CH_4 by using O_2 produced in situ from nitrite (Ettwig et al. 2010). Methanotrophs play a major role in the environment, oxidizing methane that is released into the environment due to the activity of methanogenic archaea and industrial human activity, thereby mitigating the effects of this potent greenhouse gas (Hanson and Hanson 1996). Methanotrophs of the phylum *Proteobacteria* are classified as type I or type II methanotrophs according to whether they belong to the γ- or α-class of the *Proteobacteria*, respectively. Methanotrophs can be isolated from many ecosystems including soils, peatlands, rice paddies, sediments, freshwater and marine systems, alkaline soda lakes, acidic hot springs, mud pots, cold ecosystems, and tissues of higher organisms (McDonald et al. 2008).

Within the *Gammaproteobacteria*, there are currently 16 genera of methanotrophs within the family *Methylococcaceae* and 3 genera in the family

Methylothermaceae (Table 1). The genera within the family *Methylococcaceae* are *Methylobacter*, *Methylocaldum*, *Methylococcus*, *Methylogaea*, *Methyloglobulus*, *Methylomagnum*, *Methylomarinum*, *Methylomicrobium*, *Methylomonas*, *Methyloparacoccus*, *Methyloprofundus*, *Methylosoma*, *Methylosphaera*, *Methylosarcina*, *Methyloterricola*, and *Methylovulum*. *Methylohalobius*, *Methylomarinovum*, and *Methylothermus* are the methanotrophs in the *Methylothermaceae* family. All methanotroph species of this phylum are highly specialized, to the point that they grow only on methane and its one-carbon derivatives such as methanol and cannot grow on complex, multicarbon substrates such as sugars or organic acids. They assimilate carbon predominantly via the RuMP pathway. Also, filamentous gammaproteobacterial methanotrophs of the genera *Crenothrix* and *Clonothrix* have been described, but they have been observed only in enrichments (Oswald et al. 2017).

The *Alphaproteobacteria* currently include two methanotroph genera in the family *Methylocystaceae*, *Methylosinus*, and *Methylocystis* and three genera in the family *Beijerinckiaceae*, *Methylocella*, *Methylocapsa*, and *Methyloferula*. Alphaproteobacterial methanotrophs assimilate C_1 compounds via the serine pathway; they possess either the ethylmalonyl-coenzyme A pathway for glyoxylate regeneration or a glyoxylate bypass (Chistoserdova et al. 2009). *Beijerinckiaceae* representatives of methanotrophs additionally contain the complete set of genes for the function of the Calvin-Benson-Bassham (CBB) cycle. Members of the genus *Methylocella* and some members of the genera *Methylocystis* and *Methylocapsa* are facultative methanotrophs that can grow on a narrow range of multicarbon compounds (acetate and several other organic acids, ethanol, and some short-chain alkanes) in addition to methane (Dunfield and Dedysh 2014). The alphaproteobacterial marine methanotroph within the genus, *Methyloceanibacter*, capable of methane oxidation by solely the soluble methane monooxygenase, has also been described (Vekeman et al. 2016).

The non-proteobacterial methanotrophs of the phylum *Verrucomicrobia*, which constitute a relatively newly described clade of methane utilizers, include three genera: *Methylacidiphilum*, *Methylacidimicrobium*, and *Methyloacida* (Dunfield et al. 2007; Islam et al. 2008; Van Teeseling et al. 2014). These bacteria typically inhabit geothermal and acidic environments, where they thrive at pH 0.5–5 (optimum 2.0), 30–65 °C (optimum temperatures between 35 °C and 50 °C). Unlike proteobacterial methanotrophs, which prefer to assimilate carbon from reduced forms of C_1 carbon, the members of this group are autotrophs and use methane only as a source of energy, oxidizing it to CO_2 and then fixing CO_2 using the CBB cycle (Khadem et al. 2011). Finally, the methanotroph "*Candidatus* Methylomirabilis oxyfera" is a member of the deep-branching bacterial phylum NC10. It generates dioxygen from nitrite for in situ monooxygenation of methane despite being an obligate anaerobe (Ettwig et al. 2010). The complete list of methanotrophs validated today is presented on the Methanotroph Commons website http://www.methanotroph.org.

Table 1 Classification of genera of aerobic methanotrophs

Genus	Phylogeny	MMO type	C1 assimilation	ICM type[a]	N_2 fixation	G + C (mol %)	Major PLFA[b]	Trophic niche
Family *Methylococcacea*								
Methylobacter	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	49–54	16:1	Some psychrophilic
Methylosoma	*γ-Proteobacteria*	pMMO	Not known	Type I	Yes	49.9	16:1	Not extreme
Methylomicrobium	*γ-Proteobacteria*	pMMO +/− sMMO	RuMP	Type I	No	49–60	16:1	Halotolerant/ alkalophilic
Methylomarinum	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	50.9–51.7	16:1/16:0	Halotolerant
Methyloparacoccus	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	65.6	16:1/16:0/ 14:0	Not extreme
Methylomonas	*γ-Proteobacteria*	pMMO +/− sMMO	RuMP	Type I	Some	51–59	16:1	Some psychrophilic
Methylosarcina	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	54	16:1	Not extreme
Methylosphaera	*γ-Proteobacteria*	pMMO	RuMP	ND[c]	Yes	43–46	16:1	Psychrophilic
Methylococcus	γ-*Proteobacteria*	pMMO + sMMO	RuMP/CBB/ serine	Type I	Yes	59–66	16:1	Thermophilic
Methylogaea	*γ-Proteobacteria*	pMMO	RuMP	Type I	*nifH*$^+$	63.1	16:0/16:1/ 15:0	Not extreme
Methylovulum	*γ-Proteobacteria*	pMMO + sMMO	RuMP	Type I	*nifH*$^+$	49.3	16:0/14:0	Psychrotolerant
Methyloprofundus	*γ-Proteobacteria*	pMMO	RuMP	Type I	Yes	40.5	16:1/16:0/ 16:2	Psychrotolerant
Methylocaldum	*γ-Proteobacteria*	pMMO	RuMP/CBB/ serine	Type I	No	57	16:1	Thermophilic
Methyloglobulus	*γ-Proteobacteria*	pMMO	RuMP	Type I	Yes	47.7	16:1	Psychrotolerant
Methylomagnum	*γ-Proteobacteria*	pMMO + sMMO	RuMP/CBB	Type I	No	63–64	16:1/16:0/ 14:0	Not extreme
Methyloterricola	*γ-Proteobacteria*	pMMO	RuMP/CBB	Type I	*nfH*$^+$	61	16:1/16:0	Not extreme

Family *Methylothermacea*								
Methylothermus	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	62.5	18:1/16:0	Thermophilic
Methylohalobius	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	58.7	18:1	Halophilic
Methylomarinovum	*γ-Proteobacteria*	pMMO	RuMP	Type I	No	66	18:1/16:0	Thermophilic/ halotolerant
Family *Crenothrichaceae*								
Crenothrix	*γ-Proteobacteria*	pMMO +/− sMMO	RuMP	Type I				Not extreme
Clonotrix	*γ-Proteobacteria*	pMMO		Type I				Not extreme
Family *Methylocystaceae*								
Methylocystis	*α-Proteobacteria*	pMMO +/− sMMO	Serine	Type II	Yes	62–67	18:1	Some acidophilic
Methylosinus	*α-Proteobacteria*	pMMO + sMMO	Serine	Type II	Yes	63–67	18:1	Not extreme
Family *Beyerinkiacea*								
Methylocella	*α-Proteobacteria*	sMMO	Serine	NA4	Yes	60–61	18:1	Acidophilic
Methylocapsa	*α-Proteobacteria*	pMMO	Serine	Type III	Yes	63.1	18:1	Acidophilic
Methyloferula	*α-Proteobacteria*	sMMO	CBB/serine	NA[d]	Yes	55.6–57.5	18:1	Acidophilic
Methyloceanibacter	*α-Proteobacteria*	sMMO	Serine	No	Yes	64	18:1	Not extreme
Family *Methylacidiphilaceae*								
Methylacidiphilum	*Verrucomicrobia*	pMMO	CBB	NA4	Yes	60.9–63.8	18:0	Acidophilic
Methylacidimicrobium	*Verrucomicrobia*	pMMO	CBB	type I/ NA4			18:0	Acidophilic
Methyloacida	*Verrucomicrobia*	pMMO	CBB	NA4			18:0	Acidophilic
Phylum NC10								
"Candidatus Methylomirabilis oxyfera"		pMMO	CBB					

[a]*ICM* intracellular membrane
[b]*PLFA* phospholipid fatty acid
[c]*ND* not determined
[d]NA not applicable because ICMs are very limited in this genus

2 Methane Oxidation

Methanotrophs oxidize methane to methanol in the presence of oxygen, with the production of one water molecule and an input of two electrons (Fig. 1). The reaction is catalyzed by methane monooxygenase (MMO), which exists in two structurally and biochemically distinct forms, particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO).

2.1 sMMO

sMMO comprises three components: the active site-containing hydroxylase (MMOH), a reductase (MMOR), and a regulatory protein (MMOB) which is required for efficient methane oxidation. MMOH is an $(\alpha\beta\gamma)_2$ homodimer, of which the α subunit contains a di-iron center coordinated by four glutamates, two histidines, and several water molecules. Electrons for methane oxidation are transferred from NADH to this active site of MMOH via FAD and [2Fe-2S] clusters in MMOR. The mechanism of sMMO has been reviewed in depth recently (Lawton and Rosenzweig 2016). Briefly, the di-iron(III) site of MMOH (MMOHox) is reduced by MMOR in two sequential electron transfer events to the di-iron(II) state (MMOHred). Oxygen reacts with MMOHred to form intermediate O, followed by the peroxo intermediates P*, a di-iron(II) species, and P, a peroxo-bridged di-iron (III) species that is converted to the di-iron(IV) intermediate Q, which is defined by its characteristic absorption feature at 420 nm. Intermediate Q is believed to react with methane resulting in the formation of the product complex, T (Brazeau and Lipscomb 2000; Lee et al. 1993). The structures of MMOHox and MMOHred have been determined by X-ray crystallography (Rosenzweig et al. 1993), but molecular details of the intermediates have been elusive due to their transient nature (Banerjee et al. 2015). MMOB increases the reaction rate of sMMO with dioxygen by 2–3 orders of magnitude. The binding of MMOB to MMOH alters the electronic

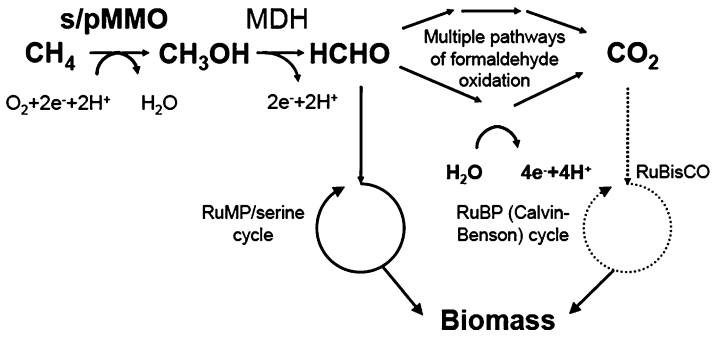

Fig. 1 Pathways of carbon metabolism in methanotrophs

structure and reduces the reduction potential of the di-iron center (Lee et al. 2013; Wang and Lippard 2014; Banerjee et al. 2015).

The facultative methanotroph *Methylocella silvestris* BL2 possesses two distinct di-iron center monooxygenase gene clusters, one encoding sMMO and the other encoding a propane monooxygenase (PrMO) (Crombie and Murrell 2014). During growth on a mixture of these gases, the strain efficiently consumes both gases at the same time. Such metabolic flexibility may be important in many environments where methane and short-chain alkanes co-occur.

2.2 pMMO

pMMO is a copper-containing, membrane-associated enzyme that consists of three polypeptides with molecular masses of 49, 27, and 22 kDa, encoded by the *pmoB*, *pmoA*, and *pmoC* genes usually organized in the operon *pmoCAB*. The enzyme has an $(\alpha\beta\gamma)_3$ stoichiometry. The N-terminus of PmoB is located in the periplasm, whereas the N-termini of PmoA and PmoC are cytoplasmic. The active site is a copper center coordinated by three histidine residues in the N-terminal periplasmic region of PmoB (Balasubramanian et al. 2010). A second metal-binding site is located in PmoC. The native reductant of pMMO may be ubiquinol generated by a type II NADH:quinone oxidoreductase. Another possibility is that methanol oxidation by methanol dehydrogenase (MDH) in the periplasm is coupled to methane oxidation, providing electrons via the electron acceptor of MDH, cytochrome c_L. pMMO and MDH encoded by the *mxa* operon form a supercomplex anchored in the intracytoplasmic membranes, and that electron transfer from the PQQ-linked MDH to pMMO in vivo may drive the oxidation of methane (Culpepper and Rosenzweig 2014; Torre et al. 2015).

Within individual genomes, *pmoCAB* operons are present often in multiple copies which can be substantially sequence divergent and might encode pMMO isozymes of alternative physiological functions. In *Methylocystis* strain SC2, the enzyme encoded by *pmoCAB2* oxidizes methane at a lower apparent *K*m than the enzyme encoded by *pmoCAB1* (Baani and Liesack 2008). Methanotrophs in the genera *Methylomonas*, *Methylobacter*, and *Methylomicrobium* also encode a sequence-divergent particulate monooxygenase (pXMO) whose genes are uniquely organized in the noncanonical form *pxmABC* and whose primary substrate could be a compound other than methane or ammonia (Tavormina et al. 2011). The growth on methanol of *Methylomicrobium album* BG8 possessing a *pxm* operon is enhanced by chloromethane (Han and Semrau 2000).

2.3 Methanobactins as Vital Chalkophores

In methanotrophs that produce both the soluble and particulate MMO, the expression of these enzymes is regulated by copper ions: pMMO is expressed during growth under high copper-to-biomass ratios, whereas the soluble form of the enzyme is

expressed when the copper-to-biomass ratio is low (Murrell et al. 2000). Copper availability in the growth medium affects many other physiological features of methanotrophs: cell wall and membrane synthesis, poly-3-hydroxybutyrate accumulation, biomass yield, and carbon conversion efficiency (Kao et al. 2004; Pieja et al. 2011). It also controls the "surfaceome," or proteins on the outer surface of the outer membrane, and the expression of several multi-*c*-type cytochromes and proteins involved in copper uptake (Bergmann et al. 1999; Karlsen et al. 2011; Shchukin et al. 2011).

One way that methanotrophs meet their high copper requirement is via the biosynthesis and release of high-affinity copper-binding compounds called methanobactins (Mbns). Mbns, termed "chalkophores" because of their ability to bind copper, are low-molecular-mass (<1,200 Da) ribosomally produced, post-translationally modified peptides (DiSpirito et al. 2016; Dassama et al. 2017). These molecules exhibit similarities to certain iron-binding siderophores but are expressed and secreted in response to copper limitation. Mbns coordinate copper via two nitrogen-containing heterocycles with associated thioamide groups and surrounded by the peptide backbone, the composition of which can vary. The posttranslational modifications of a peptide precursor include ring formation, cleavage of a leader peptide sequence, and, in some cases, addition of a sulfate group (reviewed in DiSpirito et al. 2016).

Consistent with their role in copper acquisition, Mbns have a high affinity for copper ions ($>10^{21}$ M^{-1}). Following binding, Mbns rapidly reduce Cu^{2+} to Cu^{1+}. Mbns may have a more significant role in copper uptake in situ, where copper speciation and distribution will be much more complex and will include copper associated with a wide range of organic materials (e.g., humic and fulvic acids), as well as copper found either sorbed onto or as part of various mineral phases (Fru et al. 2011). Mbns are secreted into the medium in an apo-form and transported into bacterial cells as a Cu-Mbn complex. Mbns can extract copper from insoluble minerals and could be important for mineral weathering.

In addition to binding copper, Mbns bind most transition metals and near-transition metals and protect the host methanotroph as well as other bacteria from toxic metals. In addition to Cu(II) or Cu(I), Mbns bind other metals including Hg (II), Ag(I), Au(III), Co(II), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(VI), or Zn (II) but not Ba(II), Ca(II), La(II), Mg(II), and Sr(II). However, the binding constants for other metals are lower than that for Cu(II). Therefore, the growth and activity of methanotrophs synthesizing Mbn can be accompanied by solubilization and in situ immobilization of many metals, thus decreasing their toxicity to other components of the microbial community. In particular, Mbn reduces Hg(II) into Hg(0); in this case mercury does not evaporate and remains associated with methanobactin and bound to biomass of methanotrophs. This defines the potential ecophysiological significance of Mbn-producing methanotrophs for bioremediation of ecosystems polluted with heavy metals (reviewed in DiSpirito et al. 2016).

Several Mbn biosynthesis genes and sequences encoding peptide precursors have been identified. Along with MbnA and two putative genes participating in biosynthesis – MbnB and MbnC – the operon encodes an aminotransferase, a

sulfotransferase, an FAD-dependent oxidoreductase, and transporters, including a multidrug resistance exporter and a TonB-dependent transporter (Semrau et al. 2013). Operons encoding the MbnA are present in non-methanotrophic bacteria as well, suggesting a broader role in and perhaps beyond copper acquisition. Mbn-like compounds are found in yeast mitochondria suggesting that these molecules are a more universal phenomenon. Mbn can be used for copper elimination from semiconductor industry wastes, treatment of Wilson disease, copper metabolism disorders in humans, and copper extraction from insoluble minerals (DiSpirito et al. 2016).

A new family of copper storage proteins (Csps) which provide an internal copper source when copper becomes limiting has been discovered in *Methylosinus trichosporium* OB3b (Vita et al. 2015). It possesses three Csps: Csp1 and Csp2, which have predicted twin arginine translocase-targeting signal peptides and are therefore thought to be exported after folding, as well as the cytosolic Csp3. Csp1 forms a tetramer of four-helix bundles that can bind up to 52 Cu^{+} ions via Cys residues. Switchover to sMMO is accelerated in the Δ*csp1 csp2* mutant compared to the wild type, suggesting that Mbn produced under such conditions can readily remove all Cu^{1+} from Csp1 and therefore may play a role in helping to utilize Csp1-bound copper.

3 Methanol Oxidation

Methanol dehydrogenase (MDH) catalyzes the second step in microbial methane conversion. Methanotrophs and gram-negative methanol-utilizing bacteria possess an MDH with pyrroloquinoline quinone (PQQ) at its catalytic center. This MDH belongs to the broad class of eight-bladed β-propeller quinoproteins, which comprise a range of other alcohol and aldehyde dehydrogenases. A well-studied MDH is the heterotetrameric MxaFI-MDH comprised of two large catalytic subunits (MxaF) and two small subunits (MxaI). It binds calcium as a cofactor that assists PQQ in catalysis. The purified MxaFI is most active at alkaline pH (pH 9–11) in the presence of ammonium or methylamine as activator. MxaFI exhibits wide substrate specificity to primary C_1–C_5 alcohols with the highest affinity to methanol (K_m = 20–70 μM). Oxidation of methanol is coupled with reduction of the prosthetic group PQQ to the corresponding quinol ($PQQOH_2$) followed by a two-step transfer of electrons to the acceptor, which is an inducible cytochrome c_{551} (c_L), and further via the cytochromes c_{550} (c_n) and c_{552} to the terminal oxidase (Anthony and Williams 2003).

Another MDH, known as XoxF-MDH, is often present along with MxaFI in methanotrophs and various methylotrophs as well as in some gram-negative non-methylotrophic bacteria (Chistoserdova 2011, 2016). XoxF sequences exhibit approximately 50% amino acid identity with MxaF sequences. XoxF-MDHs are homodimeric proteins lacking the small subunit and possess a rare-earth element (REE) instead of calcium. In the structure of XoxF from the thermoacidophilic methanotroph *Methylacidiphilum fumariolicum*, the ligands for binding Ln^{3+} ions can be recognized. Two of them are conserved with respect to those binding Ca^{2+} in

MxaFI-type MDH, while an additional ligand is specific to the XoxF enzyme (Khadem et al. 2012). The accommodation of an REE requires the presence of a specific aspartate residue near the catalytic site.

XoxF from *M. fumariolicum* has an extraordinary high affinity for methanol with an affinity constant as low as 0.8 μM; it does not need ammonium activation and is active at neutral pH (optimum pH of 7.0), conditions which correspond to natural condition within the cell. In all cases when tested, only the lighter lanthanides (such as La^{3+} and Ce^{3+}) support activity, while the heavier lanthanides are inactive (Pol et al. 2014; Vu et al. 2016). The presence of a suitable REE confers a superior catalytic efficiency on XoxF-MDHs. Lanthanides increase *xoxF* expression and decrease *mxa* expression, completely blocking *mxa* expression at micromolar concentrations (Chu and Lidstrom 2016). The response regulator MxaB controls transcription of the two methanol dehydrogenase genes (and its own expression) in response to lanthanides. Besides its catalytic function, a regulatory function has been suggested for XoxF. In *M. extorquens*, while removal of either of the two highly similar XoxF proteins caused no phenotype in terms of expression of the Ca^{2+}-MDH, removal of both abolished transcription from *mxaF*. However, no DNA-binding motifs are present in its sequence; thus, a more complex regulatory mechanism must be involved.

The rare-earth elements, such as lanthanum, cerium, praseodymium, and neodymium, are relatively abundant in the Earth's crust. However, lanthanide concentrations found in the environment might not be sufficient to repress *mxa* transcription (Chu and Lidstrom 2016). XoxF-MDHs are abundant in genomes of methylotrophic bacteria and also in organisms that hitherto are not known for a methylotrophic lifestyle. *xoxF*-type genes have been detected at high abundance in natural communities of lake sediments, plant phyllosphere, nutrient-rich coastal ocean waters, and plume waters off a hydrothermal vent in the deep ocean sea sponge microbiome (Delmotte et al. 2009; Mattes et al. 2013; Moitinho-Silva et al. 2014; Sowell et al. 2011).

4 Formaldehyde and Formate Oxidation

A major portion of the reducing equivalents required for methane oxygenation is formed during formaldehyde oxidation to CO_2. Three pathways for formaldehyde oxidation in methanotrophs are predicted: (1) oxidation through dye-linked heme-containing formaldehyde dehydrogenase (Patel et al. 1980), (2) tetrahydromethanopterin (H_4MPT)- and tetrahydrofolate (H_4F)-mediated C_1 transfers (Vorholt 2002), and (3) the minor dissimilatory ribulose monophosphate (dRuMP) pathway (Trotsenko and Murrell 2008). The Xox-type and Mxa-type MDHs also play at least some role in formaldehyde oxidation. Moreover, the broad-specificity aldehyde dehydrogenases have been predicted by analyses of genomes, but their role in primary C_1 oxidation remains to be validated.

In proteobacterial methanotrophs, the H_4MPT-mediated pathway generates 1 mol of NAD(P)H per 1 mol of formaldehyde converted to formate. The

H_4MPT-dependent formaldehyde conversion is initiated by the condensation of formaldehyde and the pterin cofactor to give the N^5,N^{10}-methylene derivative. The formaldehyde-activating enzyme (Fae) accelerates this spontaneous reaction in *M. extorquens* AM1, and its gene is present in all proteobacterial methanotrophs. N^5,N^{10}-methylene-H_4MPT is oxidized to N^5,N^{10}-methenyl-H_4MPT^+ by NAD(P)$^+$-dependent methylene-H_4MPT dehydrogenase, MtdB, catalyzing an essentially irreversible exergonic reaction (−13 kJ/mol). Methenyl-H_4MPT cyclohydrolase (Mch) catalyzes conversion of methenyl-H_4MPT into N^5-formyl-H_4MPT. Conversion of N^5-formyl-H_4MPT to formate is catalyzed by the formyltransferase complex, FhcA, FhcB, and FhcC, which exhibit sequence identity to the subunits FmdA, FmdB, and FmdC of formyl methanofuran (MFR) dehydrogenase from methanogenic and sulfate-reducing archaea. The H_4MPT cofactor-based pathway for C_1 transfer is not present in the genome of *M. infernorum* SolV.

Formate produced through the H_4MPT pathway may be oxidized into CO_2 by NAD^+-dependent formate dehydrogenase (FDH) or converted to methylene-H_4F, the starting substrate for the serine cycle, via the reductive pathway involving formyl-H_4F ligase (FtfL), methenyl-H_4F cyclohydrolase (Fch), and methylene-H_4F dehydrogenase (MtdA) (Crowther et al. 2008). This methylene-H_4F synthesis pathway is dominating over assimilatory flux in *M. extorquens* AM1 in contrast to the direct condensation formaldehyde with H_4F. Remarkably, Fch and MtdA enzymes present in all α- and γ-proteobacterial methanotrophs but verrucomicrobial methanotrophs do not possess methenyl-H_4F cyclohydrolase and methylene-H_4F dehydrogenase. Instead, like many other bacteria, verrucomicrobial methanotrophs apparently use the *folD* gene product to perform the same reactions.

The formate dehydrogenase (FDH) serves as a terminal enzyme in the oxidative pathway. NAD^+-dependent FDH purified from *Ms. trichosporium* OB3b consists of two types of polypeptides and functions in vitro as an electron donor for sMMO or nitrogenase (with the additional participation of ferredoxin-NAD^+ reductase and ferredoxin). However, in genomes of methanotrophs, there are from one to four open reading frames encoding FDH-like proteins, and the roles of FDH isozymes warrant further study.

The minor oxidative RuMP pathway is functioning in γ-proteobacterial methanotrophs. This route includes 3-hexulose 6-phosphate synthase (HPS), 6-phospho 3-hexulose isomerase (PHI), glucose-6-phosphate dehydrogenase (GPDH), and 6-phosphogluconate dehydrogenase (PGDH) and leads to the production of 2 mol of NAD(P)H and 1 mol of CO_2 from 1 mol of formaldehyde.

5 Carbon Assimilation Pathways

Methanotrophs use three pathways for methane carbon assimilation: the serine cycle, the ribulose monophosphate (RuMP), and the ribulose bisphosphate (RuBP) cyclic pathways.

5.1 The Serine Cycle

In α-proteobacterial methanotrophs, N^5,N^{10}-methylene-H_4F is the entry point of reduced one-carbon compounds into the serine cycle (Fig. 2). N^5,N^{10}-methylene-H_4F is formed either in the spontaneous reaction of formaldehyde with H_4F or via an alternative route involving formyl H_4F ligase. N^5,N^{10}-methylene-H_4F reacts with glycine to produce serine by the action of the serine hydroxymethyltransferase (SHTM). The amino group of serine is then transferred by a specific serine-glyoxylate aminotransferase (SGAT) to glyoxylate, thus forming glycine and hydroxypyruvate. Hydroxypyruvate reductase (HPR) converts hydroxypyruvate to glycerate, and ATP-glycerate kinase phosphorylates glycerate to 2-phosphoglycerate or 3-phosphoglycerate, followed by isomerization to phosphoenol pyruvate (PEP) which is subsequently carboxylated to oxaloacetate. The reduction of oxaloacetate by malate dehydrogenase forms malate which is then converted to malyl-CoA by malate thiokinase. Finally, malyl-CoA lyase forms glyoxylate and acetyl-CoA, the latter being a primary product of the serine cycle. Consequently, SHTM, SGAT, HPR, and malyl-CoA lyase are the key and indicative enzymes of the serine cycle.

In the second part of the serine cycle, acetyl-CoA is oxidized to glyoxylate which is further (trans)aminated to glycine, so that the primary acceptor of formaldehyde is regenerated. In the obligate methanotrophs lacking isocitrate lyase (ICL$^-$ variant), glyoxylate can be regenerated via the formation of acetoacetyl-CoA and

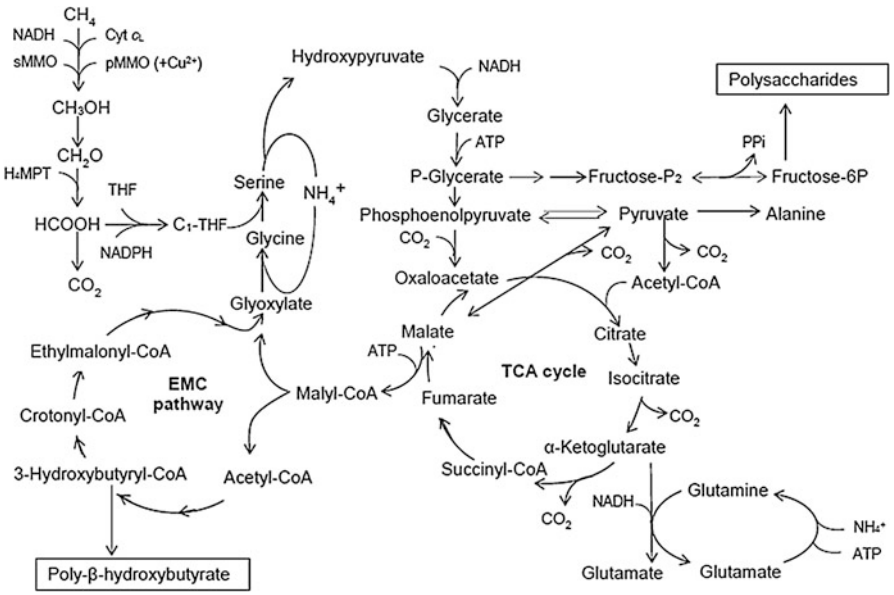

Fig. 2 Pathways of carbon metabolism in gammaproteobacterial (Type I) methanotrophs. (Modified from Rozova et al. 2015a). *TCA* tricarboxylic acid, *THF* tetrahydrofolate, *H_4MPT* tetrahydromethanopterin

hydroxybutyryl-CoA, the known intermediates of the poly-β-hydroxybutyrate biosynthesis pathway, and also crotonyl-CoA and butyryl-CoA, the intermediates of fatty acid biosynthesis. This alternate glyoxylate regeneration pathway is now known as the ethylmalonyl-CoA pathway (EMCP) analogous to that described in *Methylobacterium* (Chistoserdova et al. 2009). EMCP shares reactions and enzymes with the serine cycle (malate thiokinase, malyl-CoA lyase), the tricarboxylic acid cycle (succinate dehydrogenase, fumarase), the polyhydroxybutyrate cycle (beta-ketothiolase, acetoacetyl-CoA reductase), and other metabolic pathways (ethylmalonyl-CoA mutase, propionyl-CoA carboxylase), in addition to the specific reactions such as ethylmalonyl-CoA mutase and crotonyl-CoA reductase/carboxylase. Some methanotrophs (*Methylocella silvestris*), however, use the glyoxylate shunt instead of the EMCP (Chen et al. 2010a).

The serine cycle genes are typically located in clusters and are subject to coordinated regulation (Kalyuzhnaya and Lidstrom 2005). Interestingly, genomes of all alphaproteobacterial methylotrophs including methanotrophs, contain genes encoding phosphoribulokinase which phosphorylate ribulose-5-phosphate into ribulose-1,5-bisphosphate, regardless of the presence/absence of the functional CBB cycle. Ribulose-1,5-bisphosphate formed in the reaction is an essential metabolite for driving one-carbon assimilation via the serine pathway in *M. extorquens* AM1 (Ochsner et al. 2017).

5.2 The RuMP Cycle

The γ-proteobacterial methanotrophs assimilate methane carbon via the RuMP cycle (Fig. 3) that is initiated by a reaction catalyzed by 3-hexulosephosphate synthase (HPS) where formaldehyde is fixed with ribulose-5-phosphate to form (D-arabino)-3-hexulose-6-phosphate. This very unstable product is rapidly isomerized to fructose-6-phosphate by 6-phospho-3-hexulose isomerase (PHI). In the sequenced genomes of γ-proteobacterial methanotrophs, there are from one to three operons consisting of *hps* and *phi* genes, and several species contain an additional *hps-phi* fused gene. HPS purified from *M. capsulatus* Bath has a high molecular mass (6 × 49 kDa subunits) that corresponded to the product of the *hps-phi* fused gene possessing synthase but is lacking in isomerase activity (Ferenci et al. 1974). The recombinant HPS obtained from *Methylomicrobium alcaliphilum* 20Z by expression of *hps* is a homodimeric enzyme (2 × 40 kDa) inhibited by AMP and ADP (Rozova et al. 2017). The recombinant bi-domain HPS-PHI protein of strain 20Z has low synthase activity but no isomerase activity. Disruption of the *hps-phi* fused gene did not affect the growth rate of the mutant *M. alcaliphilum*. Interestingly, the genomes of all RuMP pathway methylotrophs which are unable to grow on methane have no HPS-PHI fused proteins. The significance of the HPS-PHI bi-domain protein in methanotroph remains to be clarified. In contrast to HPS, the characteristics of PHI are poorly documented in methanotrophs.

In the second part of the RuMP cycle, phosphohexoses are converted into C3 molecules via three mechanisms: the Entner-Doudoroff (ED) pathway,

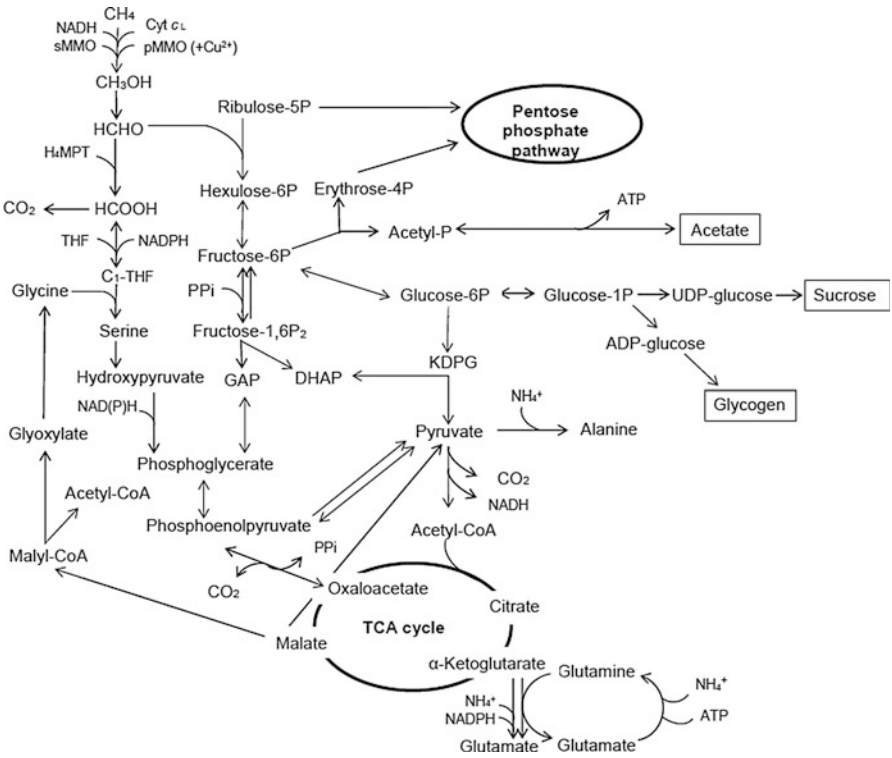

Fig. 3 Pathways of carbon metabolism in alphaproteobacterial (Type II) methanotrophs (Modified from Rozova et al. 2015a). *EMC* ethylmalonyl-CoA, *TCA* tricarboxylic acid, *THF* tetrahydrofolate, *H_4MPT* tetrahydromethanopterin

Embden-Meyerhof-Parnas (EMP) glycolysis, and phosphoketolase pathway. In the ED pathway, fructose-6-phosphate is transformed via glucose-6-phosphate and 6-phosphogluconate into 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is subsequently cleaved by KDPG-aldolase to pyruvate and glyceraldehyde-3-phosphate (GAP). However, some γ-proteobacterial methanotrophs do not have ED-encoding genes.

In the EMP pathway, fructose-6-phosphate is phosphorylated into fructose-1,6-bisphosphate (FBP) by pyrophosphate-dependent 6-phosphofructokinase (PP_i-PFK). FBP aldolase cleaves FBP to GAP and dihydroxyacetone phosphate. In this pathway, the energy of PPi, a waste product of anabolic reactions, such as the synthesis of lipids, carbohydrates, proteins, and nucleic acids, is reutilized. Reutilization of PP_i in the PP_i-mediated glycolytic pathway significantly increases the predicted efficiency of one-carbon assimilation. Conversion of phosphoenolpyruvate into pyruvate is catalyzed by a pyruvate kinase that has unusual regulatory properties. The RuMP pathway intermediates such as glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, ribulose-5-phosphate, or erythrose-4-phosphate

stimulate activities of the enzyme from *M. alcaliphilum* 20-fold, whereas ATP, PP_i, and P_i strongly inhibit the enzyme activity. Another PPi-dependent glycolytic enzyme, pyruvate-orthophosphate dikinase (PPDK), is encoded in genomes of methanotrophs facilitating full reversibility of the EMP pathway and its higher energetic efficiency in comparison to the classical glycolysis pathway. The functionality and predominance of the EMP pathway have been corroborated by transcriptomic studies, metabolomics, and ^{13}C-label distribution analysis (Kalyuzhnaya et al. 2013).

The third mechanism for phosphosugar cleavage in γ-methanotrophs involves phosphoketolase (Xfp) splitting fructose-6-phosphate (or xylulose-5-phosphate) into erythrose-4-phosphate (or glyceraldehyde-3-phosphate) and acetyl-phosphate. The reversible acetate kinase coded by the gene *ack* catalyzes ATP and acetate synthesis from acetyl-phosphate. The acetate kinase from *M. alcaliphilum* was 20-fold more active in the reaction of acetate and ATP synthesis compared to acetate phosphorylation and had a remarkably higher catalytic efficiency in this direction (Rozova et al. 2015b). In *M. alcaliphilum* 20Z, the *xfp* and *ack* genes are co-transcribed, and this phosphoketolase pathway is 1.5 times more efficient in anaerobic ATP generation compared to the classical glycolysis.

Besides methanotrophs, most aerobic methylotrophs, chemoautotrophs, and cyanobacteria, i.e., microbes whose carbon assimilation proceeds by de novo formation of the C–C bond, possess the *ack* and *xfp* genes. The phosphoketolase pathway contributes to C_2-compound production and bypasses the traditional glycolytic mechanism involving pyruvate dehydrogenase, thus preventing disruption of the C–C bond. Acetate accumulation during formaldehyde fermentation in cultures of *M. alcaliphilum* 20Z grown in microaerobic conditions suggests high metabolic flexibility that gives an advantage to gammaproteobacterial methanotrophs to survive in oxygen-limiting ecosystems. The biotechnological potential of the Xfp-based synthetic pathways has been highlighted in a recent publication (Bogodar et al. 2013).

In the third part of the RuMP cycle, the primary acceptor of formaldehyde, ribulose-5-phosphate, is regenerated from glyceraldehyde-3-phosphate and fructose-6-phosphate in a series of transaldolase/transketolase reactions analogous to those in photo- and chemotrophic bacteria.

Besides the RuMP cycle machinery, the key enzymes of the serine pathway, SHTM, SGAT, HPR, and malyl-CoA lyase, are encoded by genomes of all gammaproteobacterial methanotrophs sequenced so far, while the respective glyoxylate regeneration mechanism is not encoded (But et al. 2017).

6 CBB Cycle

The methanotrophs of the phyla *Verrucomicrobia* and NC10 are autotrophic bacteria using the CBB cycle of CO_2 fixation and using methane only as an energy source (Fig. 1). In contrast, in methanotrophs of the genera *Methylococcus* and *Methylocaldum*, three simultaneous pathways of C_1 assimilation are probably

functioning (Trotsenko and Murrell 2008). In these thermophilic/tolerant methanotrophs, the CBB cycle is a minor pathway whose contribution for C_1 assimilation remains poorly understood. The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) of *Mc. capsulatus* Bath has an $\alpha_6\beta_6$ structure which differs from the typical $\alpha_8\beta_8$ structure of the Form I RuBisCO found in *Proteobacteria*, *Cyanobacteria*, and higher plants. The genes encoding the large subunit (*cbbL*) and small subunit (*cbbS*) and putative regulatory gene (*cbbQ*) are colocated in one cluster. In *Mc. capsulatus* Bath and *Methylocaldum szegediense* O-12, RuBisCO activity is enhanced in response to temperature increase, thus providing a means for dissipation of excess heat energy (Eshinimaev et al. 2004).

7 Nitrogen Assimilation

The ability to utilize atmospheric nitrogen (N_2) as a sole nitrogen source has been experimentally proven in a number of proteobacterial methanotrophs as well as verrucomicrobial representatives (Murrell and Dalton 1983; Khadem et al. 2010). The genomes of many methanotrophs have a complete set of genes necessary for N_2 fixation which encompass a gene cluster for iron-molybdenum-dependent nitrogenase (*nifH*, *nifD*, and *nifK*) and two additional clusters that contain genes for biogenesis of cofactors and electron transfer proteins, as well as a Mo/Fe nitrogenase-specific transcriptional regulator NifA. *M. fumariolicum* SolV is able to fix N_2 under low oxygen concentration (0.5% O_2 saturation) in chemostat cultures at a dilution rate of 0.017 h^{-1}. The nitrogenase of *M. fumariolicum* SolV is extremely oxygen sensitive compared to those from the proteobacterial methanotrophs studied to date.

Methanotrophs use ammonium, nitrates, and nitrites and often can grow in the presence of urea or some amino acids as nitrogen source. Gammaproteobacterial methanotrophs assimilate NH_4^+ mainly by reductive amination of pyruvate and/or α-ketoglutarate, whereas alphaproteobacterial methanotrophs use the glutamate cycle, i.e., glutamine synthetase (GS) and the glutamine-oxoglutarate amidotransferase (GOGAT) system. The GS purified from *Mc. capsulatus* Bath is regulated by (de)adenylylation mechanisms. At concentrations of >0.5 mM NH_4^+ in the medium, GS exists in the non-active adenylylated form. Regulation of *gln*A in this methanotroph is analogous to that in enterobacteria and occurs via the Ntr system. In *Mc. capsulatus* Bath and other gammaproteobacterial methanotrophs grown on medium containing ammonia, the reductive amination of pyruvate (via alanine dehydrogenase) and/or 2-oxoglutarate (via glutamate dehydrogenase) occurs under high-ammonia growth conditions. In contrast, when grown under N_2-fixing conditions, i.e., under ammonium limitation or on medium containing nitrate, the methanotrophs assimilate ammonia via the glutamate cycle (reviewed in Trotsenko and Murrell 2008). *M. infernorum* V4 can fix ammonia both through the glutamine synthesis reaction and through the carbamoyl-phosphate synthesis reaction. The latter substrate is used in the urea cycle, for which all genes are present except for the gene encoding arginase, which cleaves arginine into urea and ornithine.

However, *M. infernorum* V4 encodes 4-aminobutyrate aminotransferase ArgD that can ultimately supply ornithine back to the cycle through a part of the TCA cycle and glutamate synthesis. Other methylotrophs possess neither arginase nor ArgD. Four predicted ammonium transporters have been identified in the genome of *Mc. capsulatus* Bath. Methanotrophs also possess genes encoding assimilatory nitrate reductase.

8 Assimilation of Methylated Amines

Among methanotrophs, *M. silvestris* BL2 is able to grow on mono- or trimethylamine as a sole source of energy, carbon, and nitrogen. Also, trimethylamine N-oxide (TMAO) can serve as nitrogen source for this bacterium. Monomethylamine is metabolized via methylated amino acids in a metabolic pathway where γ-glutamylmethylamide (GMA) and N-methylglutamate (NMG) are intermediates (Chen et al. 2010b). However, methylamine dehydrogenase is not detectable. One of the key enzymes in this pathway, GMA synthetase, catalyzes the ATP-dependent condensation of methylamine and glutamate, and a second enzyme, NMG dehydrogenase, delivers C_1 unit from NMG into the serine cycle. Mutation of the GMA synthetase gene, *gmas*, abolishes growth of *M. silvestris* on methylamine. Eight genes of this pathway are co-transcribed as an operon.

The flavin-containing trimethylamine monooxygenase (Tmm) oxidizes trimethylamine to trimethylamine N-oxide (TMAO) in *M. silvestris* BL2 (Chen et al. 2011). The Tmm contains the conserved sequence motif (FXGXXXHXXXF/Y) and typical domains for binding flavin adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. TMAO demethylase (Tdm) catalyzes demethylation of TMAO to formaldehyde and dimethylamine (Zhu et al. 2014). It is a novel Zn^{2+} and Fe^{2+}-dependent metalloprotein with the Zn^{2+}/Fe^{2+}/Tdm monomer ratio 1/1/1. It has been hypothesized that the oxygen atom is transferred from the substrate TMAO to produce formaldehyde via the high-valent iron-oxo intermediate (e.g., Fe(IV)-oxo, Fe(V)-oxo) (Zhu et al. 2016). Hexameric Tdm of *M. silvestris* BL2 has a high affinity for TMAO ($Km = 3.3$ mM; $Vmax = 21.7$ nmol min^{-1} mg^{-1}). It carries out an unusual O_2-independent oxidative demethylation utilizing the substrate TMAO as a surrogate oxygen donor. Tdm of *M. silvestris* BL2 and eukaryotic Tdms have contrasting characteristics and no sequence homology.

9 TCA Cycle

Since methanotrophs are able to obtain energy from the oxidation of reduced C1 compounds, the TCA cycle cannot be an obligatory means of energy generation. Due to low or zero activity of 2-oxoglutarate dehydrogenase in the α- and γ-proteobacterial methanotrophs, it has been assumed that the major function of the TCA cycle is to provide precursors for biomass synthesis in methanotrophs. Interestingly, the genome of the γ-proteobacterial methanotroph *Methylomicrobium*

buryatense 5GB1 encodes three pathways for conversion of 2-oxoglutarate to succinyl-CoA or succinate: a classic 2-oxoglutarate dehydrogenase complex, 2-oxoglutarate ferredoxin oxidoreductase, and a pathway through succinate semialdehyde catalyzed by 2-oxoglutarate oxidase. All these sets of genes are expressed at levels similar to other TCA cycle genes. Moreover, operation of a complete, oxidative TCA cycle contributing about 45% of the total flux for de novo malate production has been revealed in *M. buryatense* 5GB1 by mutant analysis and monitoring of ^{13}C-labeling patterns of metabolites in core metabolism (Fu et al. 2017). In *M. buryatense* 5GB1, the TCA cycle generates some energy for biosynthesis.

In *Ms. trichosporium* OB3b, the TCA cycle enzyme malate dehydrogenase exhibits a remarkably higher catalytic efficiency in vitro for reduction of oxaloacetate than for oxidation of malate (Rozova et al. 2015a). The enzyme therefore allows the products of primary C_1 assimilation to be converted to malate, a central intermediate of the serine pathway. In contrast, malate dehydrogenase from *M. alcaliphilum* 20Z displays about twofold higher catalytic efficiency toward malate oxidation over oxaloacetate reduction. These catalytic properties are in accordance with a high demand of the halotolerant bacterium for aspartate, which is a precursor of the osmoprotective compound ectoine. Overall, the biochemical properties of these two malate dehydrogenases suggest that the TCA cycle in obligate methanotrophs fulfills a predominantly anabolic function.

10 Osmoadaptation Mechanisms

Methanotrophs inhabiting saline environments such as saline lakes and marine waters include species of the genera *Methylomicrobium* (*M. alcaliphilum*, *M. buryatense*, *M. kenyense*, and *M. japanense*), *Methylobacter* (*M. marinus*), and *Methylohalobius* (*M. crimeensis*). These halotolerant or halophilic methanotrophs synthesize and accumulate in their cytoplasm the cyclic amino acid ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid). Along with ectoine, *M. alcaliphilum* accumulates enhanced levels of glutamate and sucrose, the total intracellular concentration of which balances the external osmotic pressure. *M. alcaliphilum* 20Z is tolerant to 1.5 M NaCl and accumulates ectoine at up to 20% of cell dry weight and is a potential producer of this bioprotective compound for biotechnology. As in other halophilic bacteria, methanotrophs synthesize ectoine from aspartate and acetyl-CoA using three specific enzymes: diaminobutyric acid (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA), and ectoine synthase (EctC). In *M. alcaliphilum* 20Z, the ectoine biosynthetic genes are organized in the *ectR-ectABC-ask* operon, which also encodes aspartokinase as well as a MarR-like negative transcriptional regulator EctR (Reshetnikov et al. 2011).

The genome of the moderately halophilic methanotroph *Methylohalobius crimeensis* 10Ki encodes diverse genetic systems for osmotolerance, which

include the *ectABCD* genes for ectoine and hydroxyectoine synthesis; a gene encoding a high-affinity importer of choline/glycine betaine driven by a sodium-motive force; three gene copies for choline dehydrogenase and a gene 40% identical to betaine aldehyde dehydrogenase from *Bacillus subtilis*, indicating possible glycine betaine synthesis from choline; and a pathway for sucrose synthesis and degradation/reutilization, including genes for sucrose-phosphate synthase, sucrose synthase, and fructokinase. Na^+ export and use of a sodium-motive force is suggested by the presence of genes encoding a putative Na^+/H^+ antiporter localized within an ATP synthase-encoding gene cluster and a complete *nqr* gene cluster encoding Na^+-pumping NADH:quinone oxidoreductase (Sharp et al. 2015).

A mildly acidophilic, obligate methanotroph *Methylocapsa palsarum* NE2 harbors genes indicative of aerobic anoxygenic photosynthesis. This array of genes is highly similar to that in many plant-associated *Methylobacterium* species and includes genes encoding the light-harvesting complex (*pufABCML*), the reaction center (*puhA*), as well as genes involved in biosynthesis of bacteriochlorophyll and carotenoids (Miroshnikov et al. 2017). It remains to be seen if these genes are expressed under environmental conditions.

11 Methanotrophs and Biotechnology

Methanotrophs are natural systems for the attenuation of methane emission and unique systems for methane-based bioconversions. The industrial potential of methanotrophs has been tested for the production of single-cell protein, polyhydroxyalkanoates, and lipids (potential biofuels) with methane as a feedstock for large-scale cultivation (Helm et al. 2006; Zuniga et al. 2013). Methanotrophs can generate components for nanotechnology applications (surface layers and nanoparticles), growth media, vitamin B_{12}, and soluble metabolites (methanol, formaldehyde, organic acids, and ectoine) using methane as their carbon source. Other cell components, such as metal-chelating proteins (methanobactins), enzymes (methane monooxygenases), or heterologous proteins, hold promise as future products (DiSpirito et al. 2016; Strong et al. 2016). Advances in genetic methods for methane oxidizers have made possible the use of these bacteria as hosts for production of recombinant and heterologous proteins and low-molecular-mass products (de la Torre et al. 2015; Kalyuzhnaya et al. 2015; Kalyuzhnaya 2016). Genetically engineered methanotrophs can overproduce naturally occurring metabolites or non-native compounds, including such molecules as carotenoids, isoprene, 1,4-butanediol, farnesene, or lactic acid (Henard et al. 2016; Saville et al. 2014). Scenarios for generating multiple products from a single methanotroph are presented in the recent excellent publications (Gilman et al. 2015, 2017; Levett et al. 2016). A number of methane-driven transformations, such as epoxidation, calcium carbonate precipitation, bioleaching, and bioremediation, have also been explored (Jiang et al. 2010; Strong et al. 2015, 2016; Eswayah et al. 2017).

12 Prospects for the Future

Our understanding of methanotroph diversity and methane oxidation mechanisms has changed dramatically during the last 20 years. New methanotroph strains with potentially novel metabolism have inspired new ideas for valorization of methane conversions and have stimulated new research into use of methanotrophs in biotechnology. Improved genetic systems together with highly active homologous expression systems for methanotrophs should allow metabolic engineering of methanotrophs, thus facilitating their considerable biotechnological potential. Structure-function studies on the sMMO will reveal more about the active site of this remarkable enzyme, and mutagenesis will enable its catalytic utility to be extended for the production of chiral alcohols and epoxides and degradation of larger aromatic compounds, particularly polyaromatic hydrocarbons. Another goal should be the expression of high-value heterologous proteins during the production of single-cell protein, thereby increasing the economic viability of large-scale bacterial fermentations during growth on methane. Isolation and characterization of new methanotrophs that can grow, for example, at high or low temperatures and more extreme pH may improve the potential use of methanotrophs in bioremediation and biocatalysis applications.

Another much neglected area of research on methanotrophs is the study of membrane biogenesis. Despite their observation over 40 years ago, there is still much debate as to the exact function of the intracellular membranes of methanotrophs, and they provide an excellent subject for future study. The obligate versus facultative nature of methanotrophs can also be addressed more systematically through postgenomics, as can the pathways of formaldehyde assimilation and dissimilation. Proteomic analyses will provide a wealth of information for studying the regulation of methane oxidation and may provide insights into how methane oxidation is regulated under differing environmental conditions.

Genome-wide transcriptomic studies, metabolomics, and ^{13}C-label distribution analysis with methane-grown cultures will help us to understand regulation of metabolism in methanotrophs. Molecular biological and biochemical studies are needed to answer the long-standing question as to why obligate methanotrophs grow only on methane (and other one-carbon compounds) since they possess genes encoding putative membrane transport systems for organic acids and sugars. Future postgenomic studies, using methanotroph genome sequence information as a blueprint for hypothesis testing, will undoubtedly lead to further advances in our knowledge of the biology of these fascinating bacteria and allow further exploitation in biotechnology.

13 Research Needs

More detailed physiological, biochemical, and molecular studies on the carbon assimilation and dissimilation pathways in obligate methanotrophs, especially newly isolated strains.

Functional analysis of the genomes of newly isolated methanotrophs; transcriptomic and proteomic studies to investigate gene regulation in obligate methanotrophs.

Synthetic biology of aerobic methanotrophs to engineer new pathways for production of commodity chemicals.

Development of genetic techniques for key genera of aerobic methanotrophs in order to study environmental regulation of carbon and nitrogen metabolism.

Environmental regulation of facultative methanotrophs and their growth on the various components of natural gas, methane, ethane, and propane.

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