Metabolic Features of Aerobic Methanotrophs: News and Views

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https://doi.org/10.21775/cimb.033.085

Abstract

This review is focused on recent studies of carbon metabolism in aerobic methanotrophs that specifically addressed the properties, distribution and phylogeny of some of the key enzymes involved in assimilation of carbon from methane. These include enzymes involved in sugar synthesis and cleavage, conversion of intermediates of the tricarboxylic acid cycle, as well as in osmoadaptation in halotolerant methanotrophs.

Introduction

Methanotrophs inhabit a variety of ecosystems including soils, fresh and marine waters and sediments, saline and alkaline lakes, hot springs, rice paddies, peatlands, and tissues of higher organisms. They play a major role in both global carbon and global nitrogen cycles (Hanson and Hanson, 1996; McDonald *et al.*, 2008, Ettwig *et al.*, 2010; Khadem *et al.*, 2010). The metabolic potential of methanotrophs as industrial platforms for bioconversion of methane into value added compounds is currently under intense investigation, aimed at organisms that are best suited for such applications. The recent genomic and biochemical studies demonstrate high metabolic flexibility of methanotrophs, and high specialization of the enzymes of their central metabolic pathways towards methane utilization and also towards extreme condition adaptations. From the multitude of the characterized methanotrophs, halotolerant representatives are especially promising for industrial applications, as they are genetically tractable due to the availability of a variety of genetic manipulation tools (Kalyuzhnaya *et al.*, 2015; Mustakhimov *et al.*, 2015; Fu and Lidstrom, 2017; Garg *et al.*, 2018). The adaptation of halo- and thermotolerant methanotrophs to extreme environmental conditions includes the acquisition of the specific mechanisms for synthesis and reutilization of the compatible solutes. This review focuses on the recent studies of carbon metabolism in three model species of the aerobic methanotrophs *Methylomicrobium alcaliphilum* 20Z, *Methylosinus trichosporium* OB3b, and *Methylococcus capsulatus* Bath.

The carbon assimilation pathways

Methanotrophs obtain energy for growth predominantly via oxidation of methane to CO_2 and assimilate carbon at the level of formaldehyde, formate and/or CO_2 via three biochemical pathways that have been originally deciphered by Professor J. R. Quayle and colleagues in the middle of the 20th century. For more details, we refer the reader to the recent reviews (Bowmann, 2006; Kelly *et al.*, 2014; Webb *et al.*, 2014). The known aerobic methanotrophs belong to the phyla Proteobacteria, Verrucomicrobia, and the yet unnamed NC10 phylum. The proteobacterial methanotrophs are

classified as type I or type II, according to whether they belong to the Alpha- or Gammaproteobacteria class. The consensus is that gammaproteobacterial methanotrophs, represented by the families *Methylococcaceae, Methylothermaceae* and *Candidatus* family *Crenotrichaceae,* assimilate formaldehyde via the ribulose monophosphate (RuMP) pathway, where hexosephosphates are the early products formed by the condensation of formaldehyde and ribulose-5-phosphate. The alphaproteobacterial methanotrophs of the families *Methylocystaceae* and *Beijerinckiaceae* assimilate carbon from methane via the serine pathway, where the amino acid serine is firstly formed. The methanotrophic representatives of the Verrucomicrobia (family *Methylacidiphilaceae*) and the NC10 phyla use methane as the energy source and fix carbon at the CO_2 level, via the Calvin–Benson–Bassham (CBB) cycle (Dunfield *et al.*, 2007; Islam *et al.*, 2008; Khadem *et al.*, 2011; van Teeseling *et al.*, 2014; Rasigraf *et al.*, 2014). In this chapter we will concentrate on the heterotrophic methanotrophs that use reasonably efficient RuMP or serine cycles.

The serine cycle

Genomic analysis revealed that the genes encoding the enzymes of the serine cycle occur in both type I and type II methanotrophs, though the functionality and the role of the serine cycle in type I methanotrophs remain not fully understood (But *et al.*, 2017). In both alpha- and gammaproteobacterial methanotrophs, genes encoding hydroxypyruvate reductase (*hpr*), serine-glyoxylate aminotransferase (*sga*), glycerate-2-kinase (*gck2*), malate thiokinase (*mtkAB*), malyl-CoA-lyase (*mcl*), malate dehydrogenase (*mdh*), serine-transhydroxymethylase (*glyA*), as well as methylene tetrahydromethanopterin/methylene tetrahydrofolate (H_4F) dehydrogenase (*mtdA*) and formate-tetrahydrofolate lygase (*ftfL*) are adjacently located in a single, two or several clusters (Fig. 4.1), thus corroborating the 'module' organization of the serine cycle genes in methylotrophs (Chistoserdova, 2011). In all type II methanotrophs, the cluster additionally contains genes encoding phosphoenolpyruvate carboxylase (*ppc*) and methylene H_4F cyclohydrolase (*fch*), whereas among type I methanotroph genomes, only the *Methylomonas* species harbour the *ppc* genes. It is possible that other representatives of type I methanotrophs possess non-homologous enzymes which can perform PEP carboxylation, one possibility being a PPi-dependent PEP carboxykinase (Chiba *et al.*, 2015; Khmelenina *et al.*, 2018). Among the methanotrophs, the glyoxylate regeneration cycle associated with the poly-β-hydroxybutyrate (PHB) pathway is present only in members of the *Methylocystaceae* family, whereas in the *Beiyerinckiaceae* family, this function can be fulfilled by the glyoxylate shunt (Chen *et al.*, 2010; Matsen *et al.*, 2013). However, gammaproteobacterial methanotrophs presumably lack this pathway.

Two key serine cycle enzymes, hydroxypyruvate reductase (Hpr) and serine-glyoxylate aminotransferase (Sga), have been purified from three methanotrophic bacteria: *Mm. alcaliphilum* 20Z, *Mc. capsulatus* Bath and *Ms. trichosporium* OB3b (But *et al.*, 2017, 2018a). The biochemical properties of methanotrophic Hprs differed from those of other organisms. Unlike plants or non-methanotrophic bacteria (Chistoserdova *et al.*, 1991; Ho *et al.*, 1999; Ali *et al.*, 2003), the three methanotrophic Hprs catalysed the irreversible NAD(P)H-dependent reduction of hydroxypyruvate and glyoxylate, but were unable to oxidize of glycerate and glycolate (But *et al.*, 2017). Such peculiarity, along with high activity and affinity to hydroxypyruvate, suggest that these enzymes must be of primary importance for C1 assimilation. Like other Hprs characterized to date, Hprs from *Mm. alcaliphilum* 20Z and *Mc. capsulatus* Bath are homodimeric, whereas the *Ms. trichosporium* Hpr is tetrameric (But *et al.*, 2017). The three enzymes displayed similar pH optima *in vitro,* while these strains are either neutrophilic (strains OB3b and Bath) or alkaliphilic (strain 20Z). Hpr from *Ms. trichosporium* OB3b displayed higher activity and affinity than the enzymes from *Mm. alcaliphilum* 20Z and *Mc. capsulatus* Bath. Hpr from *Mm. alcaliphilum* 20Z was shown to be a subject to allosteric regulation, its activity increasing in the presence of the intermediates of glycolysis and the TCA cycle, serine, ADP, ATP and inorganic pyrophosphate, but decreasing in the presence of Pi, pyruvate, acetyl-CoA, phosphoenolpyruvate and ribulose-5-phosphate. The capacity of the enzyme to reduce glyoxylate into glycolate, in combination with FAD-dependent glycolate oxidase (*ADVE02_v2_14253-14256*; *MCA1499-150* and *MALCv4_1676-1678*) (Ward *et al.*, 2004; Stain *et al.*, 2010; Vuilleumier *et al.*, 2012), suggests that

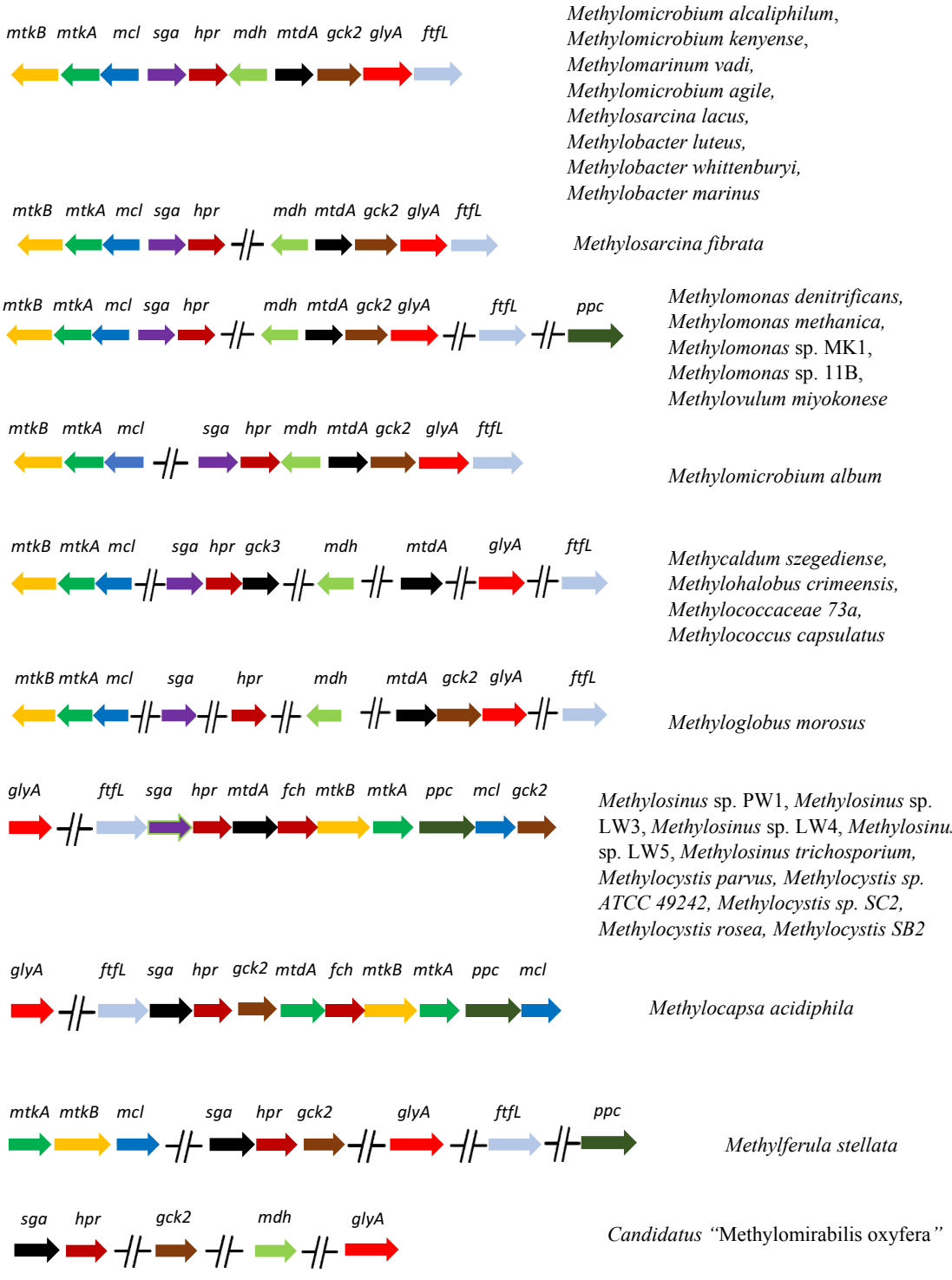

Figure 4.1 Organization of gene clusters encoding enzymes of the serine cycle in alpha- and gammaproteobacterial methanotrophs. Hydroxypyruvate reductase (*hpr*), serine-glyoxylate aminotransferase (*sga*), glycerate-2-kinase (*gck2*), glycerate-3-kinase (*gck3*), malate thiokinase (*mtkAB*), malyl-CoA-lyase (*mcl*), malate dehydrogenase (*mdh*), serine-transhydroxymethylase (*glyA*), tetrahydromethanopterine/methylentetrahydrofolate dehydrogenase (*mtdA*), formate-tetrahydrofolate ligase (*ftfL*), phosphoenolpyruvate carboxylase (*ppc*), methylene H_4F cyclohydrolase (*fch*).

Hpr could be involved in the regulation of the reduced equivalents content (But *et al.*, 2017). Phylogenetically, methanotrophic Hpr's can be divided into two divergent groups, and these share low identities with enzymes from non-methanotrophic heterotrophs (37–43%), algae (32–37%), plants (35–65%) or animals (26–46%). The phylogenetic tree of the methanotrophic Hprs implies a vertical evolution of the enzyme in the methanotrophs, with none or few instances of horizontal gene transfers (But *et al.*, 2017).

Another key enzyme of the serine cycle is serine-glyoxylate aminotransferase (Sga, EC 2.6.1.45), and these were also obtained from *Mm. alcaliphilum* 20Z, *Ms. trichosporium* OB3b and *Mc. capsulatus* Bath (But *et al.*, 2018a). The three enzymes showed similar biochemical properties, and those were also similar to the properties of Sga enzymes from other microbes and from plants (Kendziorek *et al.*, 2008; Kameya *et al.*, 2010). The methanotrophic Sgas can also transfer the amino group from serine to pyruvate, but display the highest catalytic efficiencies in the reaction between serine and glyoxylate, which would be consistent with a role in C_1 assimilation. The enzymes from strains 20Z and Bath also transferred the amino group from serine to α-ketoglutarate and from alanine to glyoxylate. Disruption of *sga* or simultaneously *sga* and *hpr* genes caused retardation of growth of *Mm. alcaliphilum* and an increase in lag-phase duration after the passage from methane to methanol. The mutant strain was shown to accumulate formaldehyde in the growth medium, suggesting that the serine cycle plays a role in type I methanotrophs, serving as one of the formaldehyde detoxification pathways (But *et al.*, 2018a).

The RuMP cycle

In the chromosomes of gammaproteobacteral methanotrophs, there are up to three copies of *hps/phi* operons coding for hexulosephosphate synthase (HPS) and phosphohexulose isomerase (PHI), key enzymes of the RuMP pathway. About half of the known type I methanotroph genomes additionally contain the fused gene *hps-phi* coding for bi-domain HPS-PHI protein (Rozova *et al.*, 2017). HPS and HPS-PHI purified from *Mm. alcaliphilum* 20Z are homodimeric enzymes (2 × 20 kDa and 2 × 40 kDa, respectively) catalysing condensation of formaldehyde with ribulose-5-phosphate with different activities (172 and 22 U/mg) and affinities to formaldehyde (K_m 0.98 mM versus 0.64 mM). AMP and ADP were shown to be powerful inhibitors for both enzymes. According to the k_{cat}/K_m ratio, HPS had a higher catalytic efficiency compared with HPS-PHI. Unexpectedly, the HPS-PHI fused enzyme lacked the isomerase activity. HPS but not the fused enzyme is prerequisite for carbon assimilation, since the insertion of a kanamycin cassette into the *hps-phi* fused gene in *Mm. alcaliphilum* 20Z did not alter growth rate of the mutant strain, whereas attempts to obtain a viable strain missing either *hps* or *phi* were unsuccessful (Rozova *et al.*, 2017). Although HPS-PHI was inactive as the isomerase, when expressed separately, both domains of the fused enzyme possessed the respective synthase and isomerase activities. The absence of the isomerase activity in the dimeric HPS-PHI could be due to the inactive enzyme configuration, since only tetrameric PHI have been characterized so far as active enzymes, whose catalytic centres were composed of four monomers (Orita *et al.*, 2005).

The unique membrane-bound hexulosephosphate synthase purified from *Mc. capsulatus* Bath by traditional chromatographic methods displayed very high activity and affinity to formaldehyde (Ferenci *et al.*, 1974). Its high molecular mass (310 kDa) implied that the bi-domain, hexameric enzyme was characterized. Importantly, besides the *hps-phi* fused gene, two complete *hps/phi* operons are present in the genome of *Mc. capsulatus* Bath (Ward *et al.*, 2004).

Surprisingly, none of the methanotroph genomes sequenced so far harbour the *hps-phi* fused gene alone. In contrast, none of the methylotrophic bacteria unable to use methane as a growth substrate encode the HPS-PHI fused enzyme. Interestingly, the genome of the model methanotroph *Methylomicrobium buryatense* possesses the *hps-phi* fused gene along with three copies of *hps/phi* operons. It remains to be demonstrated whether there is any effect of HPS multiplication on the methanotrophic growth (Rozova *et al.*, 2017). High homology between HPS and the synthase domain of the fused enzyme in the same methanotroph (73% identity in *Mm. alcaliphilum* 20Z) imply relatively recent division of HPS-PHI into separate enzymes (Rozova *et al.*, 2017). The sequences of the methanotrophic HPS comprise a tight

phylogenetic cluster, distant from those of archaea (sharing 38–40% identities), in which this enzyme functions in synthesis of pentose phosphates from C_6-phosphosugars (Yurimoto *et al.*, 2002; Mitsui *et al.*, 2003; Orita *et al.*, 2005, 2006). The bi-domain organization of the enzymes in thermophilic archaea is considered to provide enhanced stability to the enzyme against thermal inactivation (Orita *et al.*, 2005). However, such a property would not be very relevant in mesophilic bacteria.

PPi-dependent glycolysis

At least three biochemical routes for sugar cleavage can operate in gammaproteobacterial methanotrophs: the Entner–Doudoroff pathway, the glycolysis and the phosphoketolase pathway (Fig. 4.2). The cause and effect of excessive metabolic flexibility in methanotrophs is not clear. Methanotrophs obtain energy for growth predominantly from oxidation of C_1 substrates to CO_2 and can acquire ATP from oxidative phosphorylation, based on several experimental studies devoted to deciphering the respiratory mechanisms in methanotrophs (Tonge *et al.*, 1977; Chetina *et al.*, 1986). In addition, glycolysis and fermentation have been proven to be essential sources of energy in at least gammaproteobacterial methanotrophs. These methanotrophs operate a modified version of the Embden–Meyerhoff–Parnas pathway, where PPi instead of ATP serves as the phosphate donor in an energy consuming reaction of fructose-6-phosphate phosphorylation. Along the PP_i-dependent 6-phosphofructokinase (PP_i-PFK, EC 2.7.1.90), they possess another glycolytic enzyme, pyruvate phosphate dikinase (PPDK), catalysing the PPi-dependent reversible interconversion between phosphoenolpyruvate (PEP) and pyruvate, accompanied by interconversion between ATP and AMP. These substitutions make the glycolytic pathway in these organisms more efficient with respect to ATP production, compared with the classical version of the pathway. Owing to the PPi-dependent reactions, the glycolysis in methanotrophs is fully reversible. It therefore can operate as the

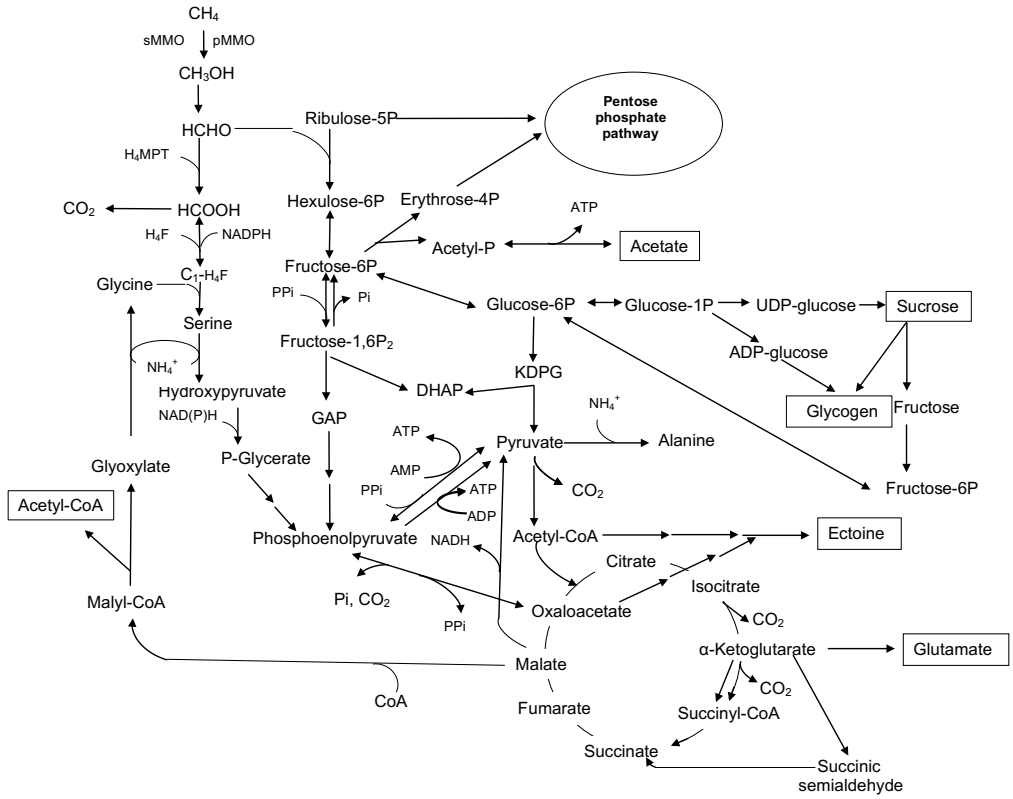

Figure 4.2 Pathways of carbon metabolism in gammaproteobacterial (Type I) methanotrophs. Modified from Rozova *et al*. (2015). H_4MPT, tetrahydromethanopterin; THF, tetrahydrofolate.

energy-inert gluconeogenic route where the energy contained in ATP can be saved in PPi molecules. In *Mm. alcaliphilum* 20Z, due to the presence pyruvate kinase, the glycolytic direction is prevailing (Kalyuzhnaya *et al.*, 2013). Considering the presence, in addition, of gluconeogenic enzymes, fructose bisphosphatase and PEP synthase, carbohydrate metabolism in type I methanotrophs is exceedingly flexible (Fig. 4.2).

PPi-PFKs of methanotrophs catalysing the reversible reaction of phosphorylation of fructose-6-phosphate (Fr6P) somewhat vary in their biochemical properties. PPi-PFK from *Methylomonas methanica* (2x45 kDa) and *Mm. alcaliphilum* 20Z (4x45 kDa) are very active enzymes that are not regulated allosterically (Reshetnikov *et al.*, 2008; Rozova *et al.*, 2010). PPi-PFK from *Mc. capsulatus* Bath is highly active as a sedoheptulose-7-phosphate (S7P) kinase, phosphorylating S7P with much higher activity than fructose-6-phosphate as a substrate. It can also phosphorylate Ru5P. In *Mc. capsulatus* Bath, which, in addition to the RuMP pathway and the serine cycle, encodes reactions of the Calvin cycle, PPi-PFK is involved in synthesis of RuBP, a primary acceptor for CO_2. Owing to modulations of activity of the ribulosebisphosphate carboxylase/oxygenase (RuBisCO) in response to temperature fluctuations (Eshinimaev *et al.*, 2004), an intracellular content of RuBP can be occasionally increased. In the case of temperature decrease or CO_2 deficiency, RuBP can be returned to the basic metabolism, retaining metabolic energy.

In at least several methanotrophs, the *pfp* gene is co-transcribed with the gene coding for a V-type H^+-pyrophosphatase (H^+-PPi-ase, EC 3.6.1.1). Therefore, direct involvement of PPi in membrane energization and ATP synthesis has been proposed (Reshetnikov *et al.*, 2008; Khmelenina *et al.*, 2018).

In alphaproteobacterial methanotrophs, the gluconeogenic function of PPi-PFK is obvious. The hexameric PPi-PFK from *Ms. trichosporium* OB3b (6×45 kDa) performs dephosphorylation of FBP with higher efficiency in comparison to phosphorylation of Fr6P (Rozova *et al.*, 2012). Unexpectedly, *Ms. trichosporium* PPi-PFK also phosphorylates S7P and Ru5P, although with much lower activities and affinities that are displayed with Fr6P. This bacterium also encodes ATP-dependent phosphoribulokinase (ID 2507407232), while it lacks the RuBisCO encoding genes. This metabolic feature implies an evolutionary link between methanotrophy and autotrophy and corroborates the recently uncovered essential regulatory functions of phosphoribulokinase and RuBP in methylotrophy (Ochsner *et al.*, 2017).

Among the methanotrophs, the representatives of the *Beijerenkiaceae* family and the phylum *Verrucomicrobia* lack PP_i-PFK (Khmelenina *et al.* 2018). In contrast, PPi-PFK is essential for methanotrophs possessing the intracytoplasmic membranes (ICM), suggesting a special function for PPi in their bioenergetics. The two oxygen-consuming processes, methane oxidation by particulate methane monooxygenase (pMMO) and oxidative phosphorylation, are both associated with the ICMs, suggesting complex regulation. At the same time, the reactive oxygen species generated by the electron transfer to pMMO (Murrell *et al.*, 2000; Kamachi and Okura, 2018) and the oxygen radicals generated from the respiratory chain may disturb membrane integrity and therefore hamper ATP synthesis or methane oxidation. The use of PPi instead of ATP as a phosphoryl donor in some cytoplasmic reactions can mitigate this bio-energetic problem. Analogous problem is faced by chemoautotrophs oxidizing NH_4^+ via ammonia monooxygenase, which is structurally and functionally similar to pMMO. This corroborates the supposition that the PP_i-PFK is specific to organisms that are energetically constrained (Mertens, 1991). The exceptions from this rule are acidophilic methanotrophs having pMMO but not possessing PPi-PFK-encoding genes, the *Methylacidiphilum* species that do not appear to form ICMs and only possess ATP-PFK (Fig. 4.3), and *Methylocapsa acidiphila* that forms ICMs but does not harbour a phosphofructokinase. The anaerobic methanotroph *Methylomirabilis oxyfera* possesses both pMMO and a very divergent PPi-PFK (Fig. 4.3), while apparently lacking ICMs (Wu *et al.*, 2012a). In this polygon-shaped bacterium able to couple anaerobic methane oxidation to denitrification, pMMO appears to be located in the cytoplasmic membrane (Wu *et al.*, 2012a,b). Further studies are needed to unravel the evolutionary history and the physiological roles of PPi and PPi-dependent enzymes in the methanotrophs.

Phylogenetically, methanotrophic PPi-PFKs belong to several divergent groups, and these share identities with the enzymes from non-methanotrophic heterotrophs and the protozoans (Fig. 4.3).

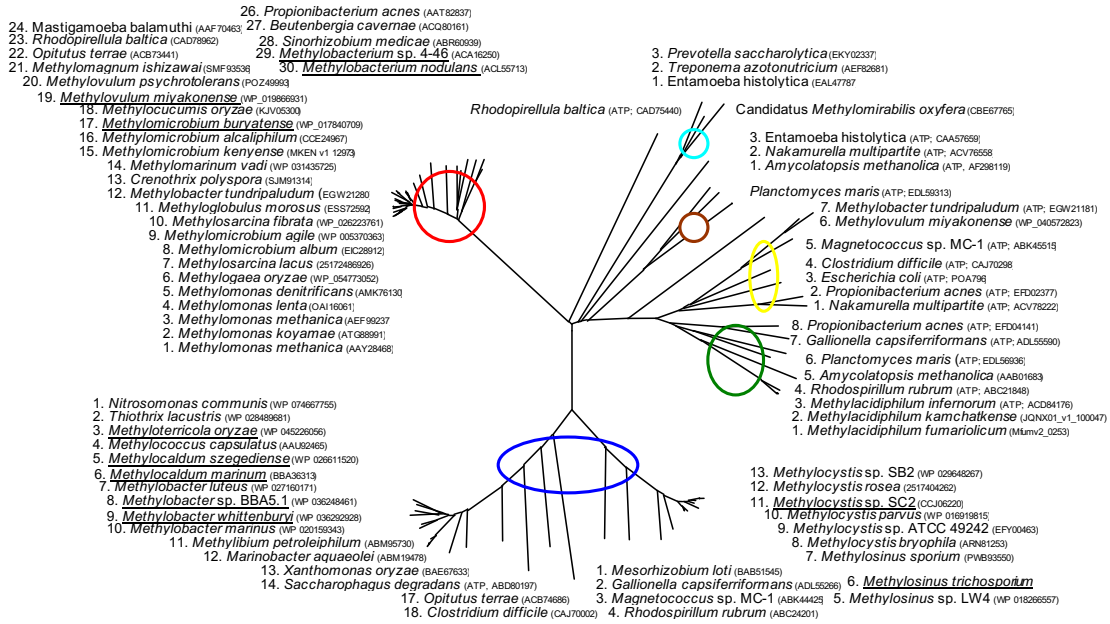

Figure 4.3 The phylogenetic tree of PPi- and ATP-dependent 6-phosphofructokinases. The phosphate donor (ATP) is indicated for ATP-PFKs in the brackets. Monophyletic groups are named according to the classification (Müller *et al*., 2001; Bapteste *et al*. 2003) as 'B2' (dark-blue), 'BI' (orange), 'III' (green), 'X' (brown), 'LONG' (blue), and 'P' (red). Amino acid sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov), the Microbial Genome Annotation and Analysis Platform (www.genoscope.cns.fr), and from the Integrated Microbial Genomes and Microbiomes system (img.jgi.doe.gov) by BLAST. The tree was constructed using the MEGA 4 program (computed from 100 independent trials).

Such distribution on the phylogenetic tree corroborates hypothesis of 'random' lateral transfer of genes coding PPi-PFKs in microorganisms (Bapteste *et al.*, 2003). Nevertheless, some correlations between the phylogenetic position and the type of carbon metabolism could also be observed.

The phosphoketolase pathway

Phosphoketolase (PKT, EC 4.1.2.9/EC 4.1.2.22) catalyses the cleavage of xylulose-5-phosphate and/or fructose-6-phosphate into acetyl-phosphate and glyceraldehyde-3-phosphate or erythrose-4-phosphate (Sánchez *et al.*, 2010). PKT-encoding genes occurs in all gammaproteobacterial methanotrophs, and at least two PKT isoforms are present in the genomes of halotolerant methanotrophs (Rozova *et al.*, 2015). The PKT pathway produces acetyl-CoA, which is the precursor of the compatible solute ectoine that is accumulated by halotolerant methanotrophs in response to increased medium salinity. The methanotroph *M. buryatense* expresses all the PKT pathway components (Henard *et al.*, 2017). In this pathway, the C_2 compounds synthesized bypass glycolysis and pyruvate decarboxylation where the C–C bond would be cleaved. The PKT pathway can produce ATP, as acetate kinase is co-expressed with PKT (Rozova *et al.*, 2015a). Acetate kinase catalyses the reversible reaction of dephosphorylation of acetyl-P into acetate with higher catalytic efficiency in the direction of ATP formation, as compared to acetate phosphorylation. Moreover, PKT in *Mm. buryatense* has proven to be involved in the global regulatory alterations of the methanotrophic metabolism (Henard *et al.*, 2017). PKT pathway engineering has been successfully used to increase yields of an array of acetyl-CoA-derived products, in diverse microbial biocatalysts. Owing to overexpression of the PKT isoform, *Mm. buryatense* has an increased biomass yields and the lipid content (Herand and Guarnieri, 2018).

In the methanotrophs, the PKT-shunted glycolytic pathway operates along with the PPi-dependent glycolysis, another energy efficient pathway, thus providing the metabolic flexibility and allowing the gammaproteobacterial methanotrophs to survive in micro-aerobic ecosystems. Numerous

environmental studies indicate that methanotrophs thrive at oxic–anoxic interfaces. The discovery of the ATP-producing assimilatory routes raised the possibility of fermentation in methanotrophs, as a new mode of methane utilization at low O_2 tensions, where formate, acetate, succinate, lactate, hydroxybutyrate and hydrogen can be the end products (Kalyuzhnaya *et al.*, 2013). This discovery challenges our understanding of methanotrophy as microbial metabolism linked solely to respiration, and it has major implications for the indispensable role of methanotrophic bacteria in removing the greenhouse gas methane in O_2-limited environments, including landfields. In such specific environments, methanotrophs drive the conversion of methane to excreted organic products and hydrogen, which are then used and transformed by non-methanotrophs.

The TCA cycle

In methanotrophs generating metabolic energy by the direct oxidation of reduced C_1 compounds or by formaldehyde fermentation, the predominantly anabolic role of the tricarboxylic acid (TCA) cycle is evident. While no or negligible activity of 2-oxoglutarate dehydrogenase has been found in methanotrophs where tested, the complete set of the genes coding for this enzyme have been identified in the genomes, and the functionality of the oxidative TCA cycle has been further proven in some methanotrophs (Matsen *et al.*, 2013; Fu *et al.*, 2017). Based on the genomic evidence, in type I methanotrophs, additional routes may bypass the need for the classic 2-oxoglutarate dehydrogenase complex, such as 2-oxoglutarate ferredoxin oxidoreductase, catalysing the reversible oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA, as well as 2-oxoglutarate oxidase + succinate semialdehyde dehydrogenase (Fu *et al.*, 2017) (Fig. 4.2). In *Mm. buryatense* 5GB1, genes for both of these enzymes are expressed at the levels similar to the ones of other TCA cycle genes.

Malate dehydrogenases (MDH, L-malate: NAD oxidoreductase, EC 1.1.1.37) purified from *Ms. trichosporium* OB3b and *Mm. alcaliphilum* 20Z displayed properties reflecting their roles in respective primary metabolisms (Rozova *et al.*, 2015b). The high catalytic efficiency of MDH from *Ms. trichosporium* OB3b in the direction of malate synthesis would be reflective of the central position of malate in the serine pathway, where malate activation to malyl-CoA and its further cleavage into acetyl-CoA and glyoxylate are key reactions in glycine regeneration. Acetyl-CoA enters the ethylmalonyl-CoA (EMC) cycle, where it is transformed to glyoxylate (Chistoserdova *et al.*, 2009) or is directed to the syntheses of fatty acids and the storage compound poly-*β*-hydroxybutyrate (PHB). The main source of OAA for the MDH reaction is carboxylation of PEP by PEP carboxylase, which is a highly active enzyme in type II methanotrophs. Some methanotrophs, such as *Ms. trichosporium* OB3b, possess two PEP carboxylases, sharing 33% identity at the amino acid level (Matsen *et al.*, 2013).

Alternatively, MDH from *Mm. alcaliphilum* 20Z shows preference for malate oxidation (OAA formation) versus malate synthesis, as follows from respective k_{cat}/K_m ratios (Rozova *et al.*, 2015b). Such enzyme features are in agreement with the high demand for aspartate in this organism, as a precursor of the osmoprotectant ectoine (Reshetnikov *et al.*, 2011).

The malic enzyme (MaE) is another enzyme that is thought to be involved in anaplerotic CO_2 fixation and malate biosynthesis. However, MaE from *Ms. trichosporium* OB3b (EC 1.1.1.40) predominantly catalyses $NADP^+$-dependent reaction of malate decarboxylation, while it has much lower activity and affinity to pyruvate (Rozova *et al.*, 2019). $NADP^+$-dependency and high efficiency in the direction of decarboxylation suggest that MaE is the 'lipogenic enzyme' important for the production of $NADPH_2$ that is needed for biosynthesis of long-chain fatty acids and/or steroids (Fig. 4.4). In contrast, *Mm. alcaliphilum* MaE (EC 1.1.1.38) catalyses non-reversible NAD^+-dependent reaction of malate decarboxylation into pyruvate but is unable to fix CO_2. Evidently, the source of malate for this reaction could be the reactions of the TCA cycle, which, in turn, can be replenished by CO_2 fixation. Although type I methanotrophs lack the PEP carboxylase (with an exception of the *Methylomonas* species), those may possess unusual PPi-dependent PEP-carboxykinases catalysing the reversible reaction of CO_2 fixation. However, the functionality and the roles of such enzymes remain to be proven. As seen in Fig. 4.5, reactions catalysed by PEP carboxykinase, MDH, MaE and PPDK can produce two PPi molecules, to trigger the modified the Emden-Meyerhof-Parnas glycolysis.

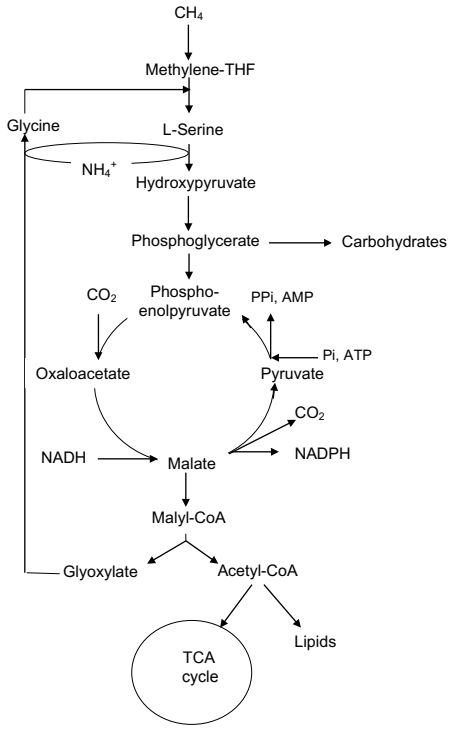

Figure 4.4 Pathways for interconversion of phosphoenolpyruvate and pyruvate in Type II methanotrophs and the role of the malic enzyme in NADPH production. TCA, tricarboxylic acid (modified from Rozova *et al*., 2019).

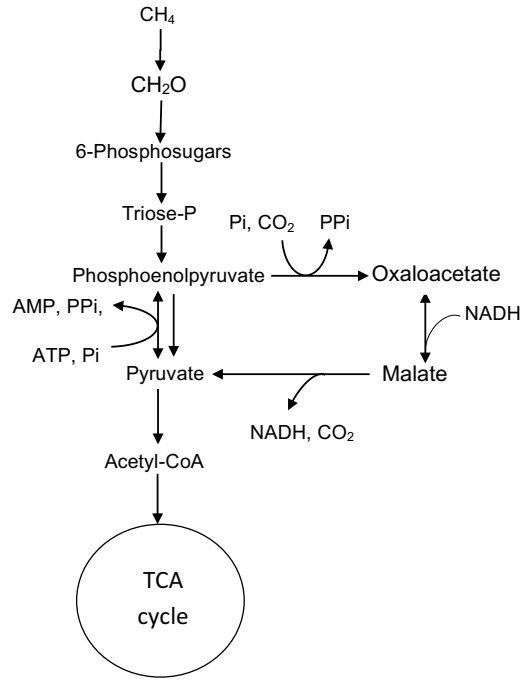

Figure 4.5 Pathways for conversion of phosphoenolpyruvate to pyruvate in Type I methanotrophs and the role of the malic enzyme in the synthesis of PPi. TCA, tricarboxylic acid.

Sucrose metabolism

The ability to synthesize sucrose is inherent in phototrophic bacteria and plants, and it has recently been revealed in non-photosynthetic bacteria, including aerobic methylotrophs that assimilate carbon via the RuMP pathway (But *et al*., 2013, 2015). In the methylotrophs, the biochemical pathway for sucrose synthesis from fructose-6-phosphate and UDP-glucose is rather similar to the one in other pro- and eukaryotic organisms, being composed of two enzymes, sucrose phosphate synthase (Sps, EC 2.4.1.14) and sucrose phosphate phosphatase (Spp, EC 3.1.3.24), with sucrose-6-phosphate as an intermediate. These enzymes are encoded by the operon *sps–spp–fruK–ams,* which also encodes, in addition to Sps and Spp, enzymes for sucrose metabolism amylosucrase (Ams, EC 2.4.1.4) and fructokinase (FruK, EC 2.7.1.4). Amylosucrase transfers the glycosyl residue from sucrose onto glycogen primer, and it also catalyses cleavage of sucrose to fructose and glucose. Glucose and fructose need to be phosphorylated by the respective ATP-dependent kinases, to enter into central metabolic pathways (But *et al*., 2013, 2015). The glucokinase from *Mm. alcaliphilum* 20Z (EC 2.7.1.1) also phosphorylates glucosamine (Mustakhimov *et al*., 2017), whereas FruK (EC 2.7.1.4) is highly specific to fructose and ATP (But *et al*., 2012). Two molecules of nucleotide triphosphate (NTP) are needed for attaching the glucosyl residue to the glycogen primer by Ams, and only one molecule of NTP is required for elongation of glycogen via glucose pyrophosphorylase (GlgC) and glycogen synthase (GlgA). Therefore, the sucrose cycle, where glycogen synthesis involves sucrose as an intermediate, could be envisaged as a dynamic mechanism that balances the intracellular levels of ATP.

Sucrose synthesis can provide some advantages to the methylotrophs. Being a carbon storage compound, sucrose also serves as a compatible solute, contributing to cell turgor maintenance at elevated salinity. However, sucrose accumulation ensures

only moderate salt tolerance by methylotrophic bacteria (But *et al.*, 2013). Of note, the genomes of most gammaproteobacterial methanotrophs isolated from soils possess the sucrose biosynthesis genes, whereas methanotrophs obtained from freshwater or sewage systems lack these genes (But *et al.*, 2015). The halotolerant methylotrophs are known to tolerate extraordinarily high concentrations of methanol, being able to grow at 7% methanol (v/v; Eshinimaev *et al.*, 2002). Since sucrose synthesis starts with early products of the RuMP pathway, high rates of formaldehyde outflow may be responsible for the methanol tolerance.

In the thermophilic *Md. szegediense* O12, sucrose synthase (Sus) is involved in metabolism of sucrose in place of Ams, cleaving disaccharide into fructose and ADP-/or UDP-glucose (But *et al.*, 2018b). The clear preference of Sus for ADP implies a connection between sucrose and glycogen metabolism, since ADP-glucose is a substrate for glycogen synthesis. So far, no genes for sucrose metabolism have been identified in methanotrophs assimilating methane via the serine pathway (phylum Alphaproteobacteria) or through the Calvin cycle (Verrucomicrobia and NC10 phyla) (But *et al.*, 2015).

Adaptation of methanotrophs to saline environments

Methanotrophs isolated from soda and hypersaline lakes, marine waters and soils are often halotolerant or halophilic, and these species belong to the genera *Methylomicrobium*, *Methylobacter* and *Methylohalobius.* All halotolerant/halophilic methanotrophs accumulate in their cells ectoine, sucrose and glutamate, which all participate in balancing osmotic pressure between cytoplasm and the cell surroundings. The cyclic imino acid ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is the major compatible solute accumulating at concentration of up to ≈1 mol per 1 l of intracellular water content. The halophilic/tolerant methanotrophs are the promising producers of ectoine, which is a multifunctional bioprotectant useful in pharmaceutical applications (Graf *et al.*, 2008). The biochemical pathway for ectoine biosynthesis in methanotrophs is almost identical to that in other halophilic prokaryotes. Three specific enzymes, diaminobutyric acid (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA, and ectoine synthase (EctC) compose this pathway encoded by the *ectABC* operon, which, in *Mm. alcaliphilum* 20Z, also includes a gene for negative transcriptional regulator EctR, of the MarR family, and a gene for an additional aspartokinase. DABA aminotransferase (EctB) from *Mm. alcaliphilum* 20Z is a hexameric pyridoxal-5′-phosphate-dependent enzyme (300 kDa) synthesizing diaminobutyric acid (DABA) from D,L-aspartyl semialdehyde using L-glutamate as a donor of an amino group. It requires K^+ for activity and stability, being more active in the presence of 0.01–0.5 M KCl (Reshetnikov *et al.*, 2011).

DABA acetyltransferase (EctA) catalyses the acetylation of DABA, yielding γ-N-acetyl-α,γ-diaminobutyric acid. The properties of EctA from *Mm. alcaliphilum* 20Z and non-methanotrophic methylotrophs *Methylophaga alcalica* ATCC 35842 and *Methylophaga thalassica* ATCC 33146 represent striking examples of bacterial eco-physiological adaptation at the enzyme level (Mustakhimov *et al.*, 2008). The EctA from the neutrophilic *M. thalassica* has optimal pH between 8.2 or 9.0, whereas the enzymes from the alkaliphilic *Mm. alcaliphilum* 20Z and *M. alcalica* have pH optima at ≥ 9.5. Activities of EctA from *M. alcalica* and *Mm. alcaliphilum* 20Z, which have been isolated from soda lakes, were increased in the presence of carbonate ions, whereas EctA from a marine isolate *M. thalassica* was considerably inhibited by carbonates. Copper ions completely inhibited the enzyme activity from *M. alcalica* but had no effect on the enzyme from *Mm. alcaliphilum* 20Z. Ectoine synthase (35 kDa) catalyses the cyclization of N-acetyl-DABA into ectoine. The methylotrophs synthesizing ectoine also encode a pathway for ectoine degradation, first proposed for a non-methylotrophic bacterium *Halomonas elongata* (Schwibbert *et al.*, 2011).

Methylohalobius crimeensis 10Ki displays the highest halotolerance recognized among the halophilic methanotrophs, and it is able to grow at 12% NaCl (Sharp *et al.*, 2015). It possesses an array of mechanisms for osmoadaptation. In addition to the *ectABCD* gene cluster, it encodes ectoine hydroxylase (EctD), an enzyme responsible for biosynthesis of hydroxyectoine. It also possesses genes for a high-affinity importer of choline/glycine betaine, driven by a sodium-motive force. It also harbours three copies of a gene for choline dehydrogenase and a gene encoding betaine aldehyde dehydrogenase, indicating possible glycine betaine synthesis

from choline. In addition, it encodes a pathway for sucrose synthesis and degradation/reutilization, which includes Sps, Spp, FruK and sucrose synthase instead of amylosucrase characteristic of other halotolerant methanotrophs. The use of a sodium-motive force and Na^+ export have been suggested by the presence of the genes encoding a putative Na^+/H^+ antiporter localized within the gene cluster encoding an ATP synthase, and a complete *nqr* gene cluster encoding Na^+-pumping NADH:quinone oxidoreductase (Sharp *et al.*, 2015).

Future trends

Despite the confirmation of the fundamental biochemical pathways for methanotrophy, which have been originally described a while ago, many important details of the methanotrophic metabolism still remain unclear. Future studies are needed to decipher the energy generation mechanisms, the basis for the obligate dependency on C1 compounds in many species, as well as the potential significance of the unusual secondary metabolites (Puri *et al.*, 2018) for central metabolism or organism's physiology. A better understanding of methanotrophy, including in the halotolerant methanotrophs that are known for robust growth, through closing the extant gaps in our knowledge, will allow for employing these organisms as novel platforms for synthetic biology applications, forming a base for microbial factories that will utilize methane, the major component of natural gas, as a feedstock for the synthesis of high-value commodity chemicals.

Acknowledgement

This material is based upon work supported by the Russian Scientific Foundation (18-04-00326).

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