Part 2

Biochemistry of Aerobic Degradation

2 Physiology and Biochemistry of the Aerobic Methane Oxidizing Bacteria

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Abstract: Methanotrophic bacteria grow aerobically using methane as a source of carbon and energy. They are widespread in the environment and play an important role in oxidizing methane in the environment, thereby mitigating the effects of global warming by this potent greenhouse gas. Methane monooxygenases (MMOs), which are the enzymes that catalyze the oxidation of methane, especially, the catalytically versatile soluble MMO, can cooxidize a wide range of hydrocarbons and chlorinated hydrocarbons, and have great potential as biocatalysts for bioremediation and biocatalysis. Methanotrophs can also be used to make single-cell protein from methane. Recent isolation of novel groups of thermophilic, acidophilic methanotrophs has revealed that these bacteria can even grow under extreme environmental conditions. The availability of genome sequences of several methanotrophs now opens up possibilities of postgenomic studies to investigate the regulation of methane oxidation in the laboratory and in the environment.

1 Introduction

Methane-oxidizing bacteria (methanotrophs) are a widely distributed group of aerobic microorganisms that use methane as their sole source of carbon and energy. They synthesize all of their cellular carbon-containing molecules from methane, and can also co-oxidize a wide range of hydrocarbons (Dalton, 2005). Anaerobic methane-oxidizing bacteria also exist but only the properties and metabolism of the aerobic methanotrophs will be described in this chapter. The majority of methanotrophs are Gram-negative bacteria that are classified as type I or type II methanotrophs according to whether they belong to the γ- or α-subdivisions of the proteobacteria, respectively. They play a major role in oxidizing methane that is produced in the environment due to the activity of methanogenic archaea, and therefore play a major role in the global methane cycle by preventing the release of much of this methane to the atmosphere, thereby mitigating the effects of this potent greenhouse gas (Hanson and Hanson, 1996). Methanotrophs contain the remarkable enzyme methane monooxygenase (MMO). MMOs oxidize methane to methanol and catalyze a very large number of adventitious oxidation reactions that are the basis of the ability of methanotrophs to carry out processes in biocatalysis and bioremediation. Thus, they have found applications in production of single cell protein (SCP) from natural gas and bioremediation of trichloroethene (TCE)-contaminated groundwater.

Methanotrophs are ubiquitous in nature and have been isolated from many environments including soils, peatlands, rice paddies, sediments, freshwater and marine systems, acidic hot springs, mud pots, alkaline soda lakes, cold environments, and tissues of higher organisms (McDonald et al., 2008). Methanotrophs can broadly be divided into Type I and Type II methanotrophs (γ- and α-proteobacteria, respectively). Genera of Type I methanotrophs include *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylosphaera*, *Methylocaldum*, *Methylothermus*, *Methylohalobius*, *Methylomicrobium*, *Methylosarcina*, and *Methylosoma* and Type II methanotrophs include *Methylosinus*, *Methylocystis*, *Methylocella*, and *Methylocapsa*. Recently two filamentous methanotrophs, *Crenothrix polyspora* and *Clonothrix fusca* as well as the first fully authenticated facultative methanotroph, *Methylocella silvestris*, which can grow on either methane or some multi-carbon compounds, have been described (Dedysh et al., 2005; Theisen and Murrell, 2005). Even more remarkable is the recent isolation of three thermoacidophilic methanotrophs which belong to the bacterial phylum *Verrucomicrobia* and thus are only distantly related to the proteobacterial methanotrophs. These three new isolates provisionally named "*Methylokorus*", "*Acidimethylosilex*," and "*Methyloacida*", grow remarkably at pH 1.5

and 65°C (reviewed in Semrau et al., 2008). The characteristics of these aerobic methanotrophs are summarized in *Table 1* and described in Chapter 26, Vol. 3, Part 1.

2 Physiology and Biochemistry of Methanotrophs

MMO, the principal defining enzyme of methanotrophs, exists in two structurally and biochemically distinct forms, particulate (pMMO) and soluble (sMMO). pMMO is a copper-containing enzyme that is associated with unusual intracellular membranes that take the form of vesicular disks in type I methanotrophs and paired peripheral layers in type II organisms. sMMO is a cytoplasmic di-iron center-containing enzyme complex (reviewed in Hakemian and Rosenzweig, 2007). Both forms of MMO, soluble (sMMO) and particulate (pMMO), can not only oxidize methane to methanol but also co-oxidize a range of hydrocarbons and chlorinated pollutants, and hence are responsible for much of the biotechnological potential of methanotrophs (Smith and Dalton, 2004). In methanotrophs that can express either form of MMO, expression is regulated by the availability of copper ions. The copper-containing pMMO is expressed during growth at high copper-to-biomass ratios and sMMO is expressed during growth under low copper-to-biomass ratios (Murrell et al., 2000). The biochemistry and molecular biology of methane monooxygenases is described Chapter 17, Vol. 2, Part 4 and will only briefly be outlined below. Methanol, the initial oxidation product of methane, is oxidized to formaldehyde by a PQQ-dependent methanol dehydrogenase (MDH) (Anthony and Williams, 2003). Formaldehyde is an important branch-point in methylotrophic metabolism and multiple pathways for metabolism of formaldehyde are common in methanotrophs. The methanotrophs possess two pathways for fixation of formaldehyde, the serine and ribulose monophosphate (RuMP) cycles, which are active in type I and type II methanotrophs, respectively (Anthony, 1982; Trotsenko and Murrell, 2008). In addition, some methanotrophs can fix carbon dioxide into biomass via the ribulose-*bis*-phosphate (RuBP) cycle and its key enzyme RuBP carboxylase/oxygenase (Rubisco).

3 Methane Oxidation

sMMO is a three-component binuclear iron center monooxygenase that belongs to a large group of bacterial hydrocarbon oxygenases known as the soluble di-iron monooxygenases. sMMO, encoded by a six-gene operon *mmoXYBZDC*, has three components: (1) a 250-kDa hydroxylase with an $(\alpha\beta\gamma)_2$ structure in which the α-subunits (MmoX) contain the binuclear iron active center, where substrate oxygenation occurs; (2) a 39-kDa NAD(P)H-dependent reductase (MmoC); (3) a 16-kDa component (MmoB) known as protein B or the coupling protein that contains no prosthetic groups or metal ions. There are X-ray crystal structures for the hydroxylase component and NMR data on the structures of protein B and the flavin domain of the reductase. The catalytic cycle of sMMO has been extensively studied and excellent progress has been made toward understanding the mechanism of oxygen and hydrocarbon activation at the di-iron center (Baik et al., 2003). The crucial reaction intermediate compound Q accumulates when the reduced (Fe^{II}-Fe^{II}) hydroxylase is reacted with O_2 in the presence of protein B. In compound Q, which is kinetically competent to oxidize methane and other substrates, the di-iron center is most likely in the diferryl (Fe^{IV}-Fe^{IV}) state. The site of substrate binding of sMMO is a hydrophobic cavity deeply buried in the α subunit of the hydroxylase. There is now a good expression system for sMMO and structural and

Table 1

Classification of genera of aerobic methanotrophs

Genus	Phylogeny	MMO type	C_1 assimilation	ICM type[a]	N_2 fixation	G + C (mol %)	Major PLFA[b]	Trophic niche
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; alkaliphilic
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/Serine	type I	Yes	59–66	16:1	Thermophilic
Methylocaldum	γ *Proteobacteria*	pMMO	RuMP/Serine	type I	No	57	16:1	Thermophilic
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
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
Methylocella	α *Proteobacteria*	sMMO	Serine	NA[d]	Yes	60–61	18:1	Acidophilic
Methylocapsa	α *Proteobacteria*	pMMO	Serine	type III	Yes	63.1	18:1	Acidophilic
Crenothrix	γ *Proteobacteria*	pMMO	ND	type I	ND	ND	ND	Not extreme
Clonothrix	γ *Proteobacteria*	pMMO	ND	type I	ND	ND	ND	Not extreme
Methylokorus	*Verrucomicrobia*	pMMO	Serine	type IV?	No	ND	ND	Thermoacidophilic
Acidimethylosilex	*Verrucomicrobia*	pMMO	RuMP?	type IV?	No	ND	C18:0	Thermoacidiophilic
Methyloacida	*Verrucomicrobia*	ND	ND	type IV?	Yes	ND	ND	Thermoacidophilic

[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

site-directed mutagenesis studies have indicated that a gating residue, Leucine 110, in the α subunit of the hydroxylase is important in determining how substrates enter and are presented at the active site (Borodina et al., 2007).

pMMO is a copper-containing, membrane-associated enzyme that consists of three polypeptides, of 49, 27, and 22 kDa, encoded by the genes *pmoB*, *A*, and *C*, respectively. The X-ray crystal structure of pMMO shows that the enzyme has an $(\alpha\beta\gamma)_3$ stoichiometry (reviewed in Lieberman and Rosenzweig, 2005). The enzyme complex contains copper ions and also a di-iron center. A powerful copper chelator, methanobactin, is used to sequester copper ions for activity and/or structural integrity of pMMO (reviewed in Balasubramanian and Rosenzweig, 2008).

The best characterized methanotrophs, *Methylococcus capsulatus* (Bath) (type I) and *Methylosinus trichosporium* OB3b (type II), can produce either form of MMO. During growth under high copper-to-biomass ratios, pMMO is expressed whereas the soluble form of the enzyme is expressed when the copper-to-biomass ratio is low (Murrell et al., 2000). Methanotrophs that possess only pMMO, such as *Methylomonas methanica* and *Methylomicrobium album* BG8 have been known for many years. Recently the facultative type II methanotrophs *Methylocella silvestris* has been shown to possess sMMO but not pMMO. The properties of MMOs have attracted interest for biotechnological applications and these are described in Section 9.

4 Methanol Oxidation

Methanol produced from the oxidation of methane is further oxidized by a periplasmic enzyme methanol dehydrogenase (MDH) which has pyrroloquinoline quinone (PQQ) as a prosthetic group (Anthony and Williams, 2003). MDH is a heterotetramer $\alpha_2\beta_2$ consisting of two large (67 kDa) and two small (8.5 kDa) subunits. Oxidation of methanol is coupled with reduction of the prosthetic group ($PQQH_2$) into the corresponding quinol ($PQQH_2$) followed by 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. The biochemistry and molecular biology of methanol oxidation has been mainly studied in the Gram-negative methanol-utilizing bacterium *Methylobacterium extorquens* (Chistoserdova et al., 2003) but it is likely that the same mechanisms operate in methanotrophs.

The components of the methanol oxidation pathway are encoded by at least 25 genes. *mxa*J, *mxa*R and *mxa*S, *mxa*D, *mxa*E, and *mxa*H are required for the formation of active MDH, while *mxa*G encodes cytochrome c_{551} and the products of genes *mxa*ACKLD are involved in insertion of PQQ and Ca^{2+}into MDH. The polypeptide encoded by *mxa*B regulates transcription of the MDH genes. Two gene clusters, *pqq*ABCDE and *pqq*FG, are involved in biosynthesis of PQQ. *pqq*A encodes the proposed precursor of PQQ, containing tyrosine and glutamate. *mxb*DM and *mxa*QE, are required for transcriptional regulation of the methanol oxidation system.

5 Formaldehyde and Formate Oxidation

Formaldehyde is the key intermediate in the linear pathway of methane oxidation to CO_2. A major portion of the reducing equivalents required for methane oxygenation is formed during formaldehyde oxidation via formate to CO_2. Methanotrophs have several enzymes

involved in formaldehyde oxidation. In *Mc. capsulatus* (Bath) expressing pMMO, formaldehyde is mainly oxidized by a particulate cytochrome-linked formaldehyde dehydrogenase (FADH). Again, the majority of recent work done on formaldehyde and formate oxidation has been with methylotrophs which use pterin cofactors, H_4F/THF and H_4MPT, for activation of formaldehyde oxidation and probably the same mechanisms operate in methanotrophs. In Type II methanotrophs, the major role of the THF-pathway enzymes is maintenance of a high concentration of N^5,N^{10}-methylene-THF, which is the primary acceptor of formaldehyde in the serine pathway of C_1 assimilation. Because of the reversibility of the reactions catalyzed, these enzymes can be regulated by the requirements of the cell either into the oxidation or assimilation of formaldehyde (Vorholt, 2002). The functions of the THF-pathway enzymes have not been elucidated in Type I methanotrophs (*Figs. 1* and *2*). The reactions of H_4MPT-dependent oxidation of formaldehyde were first found in methanogenic and sulfate reducing *Archaea* and were considered to be a specific characteristic only of these strict anaerobes, till their discovery in the aerobic facultative methylotroph *Methylobacterium extorquens* AM1. The highest activities of the H_4MPT pathway enzymes, methenyl-H_4MPT cyclohydrolase and NAD(P)-dependent methenyl-H_4MPT dehydrogenase, are found in *Mc. capsulatus* (Bath) and other methanotrophs of the α- and γ-proteobacteria. Methenyl-H_4MPT-cyclohydrolase (Mch) catalyzes conversion of methenyl-H_4MPT into N^{10}-formyl-H_4MPT.

Formate oxidation is a final step in the methane oxidation pathway. In all extant methanotrophs, an NAD^+ -dependent formate dehydrogenase (FDH) is present (reviewed in Trotsenko and Murrell, 2008). The enzyme from *Ms. trichosporium* OB3b consists of two types of polypeptides (54 and 106 kDa) and functions in vitro as an electron donor for sMMO or nitrogenase (with the additional participation of ferredoxin-NAD^+ reductase and ferredoxin). In cell extracts of many methanotrophs, a highly active phenazine methosulfate-linked FDH associated with membranes has been found. In the genome of *Mc. capsulatus* (Bath) there are open reading frames (ORFs) encoding FDH-like proteins and their role warrants further study.

6 Carbon Assimilation Pathways

The pioneering studies of Prof J.R.Quayle and colleagues (e.g., see Strom et al., 1974) revealed that methanotrophs use two major pathways for primary C_1 assimilation, the RuMP and serine cycles. In both of these cycles, phosphotrioses are synthesized from formaldehyde which is the key intermediate in carbon metabolism (Anthony, 1982).

In the first part of the RuMP cycle, formaldehyde is fixed with ribulose-5-phosphate to form (D-arabino)-3-hexulose-6-phosphate in a reaction catalyzed by 3-hexulosephosphate synthase (HPS). This very unstable product is rapidly isomerized to fructose-6-phosphate by phosphohexuloisomerase (PHI). These two specific enzymes catalyze the formation of both C-C bonds and phosphohexoses in Type I methanotrophs. In *Mc. capsulatus* (Bath), the purified HPS is a homohexameric (310 kDa) membrane-bound enzyme with an unusually large subunit size (49 kDa). Based on its gene sequence, the HPS molecular mass appears to correspond to the product of the *hps-phi*-fused gene. By comparison with HPS, the characteristics of PHI are poorly documented in methanotrophs. In the second part of the RuMP cycle, the phosphohexoses are split to (phospho)trioses. In methanotrophs this cleavage occurs by two simultaneous pathways, via the Entner-Doudoroff and Embden-Meyerhof-Parnas variants. In the first variant, fructose-6-phosphate is converted via glucose-6-phosphate

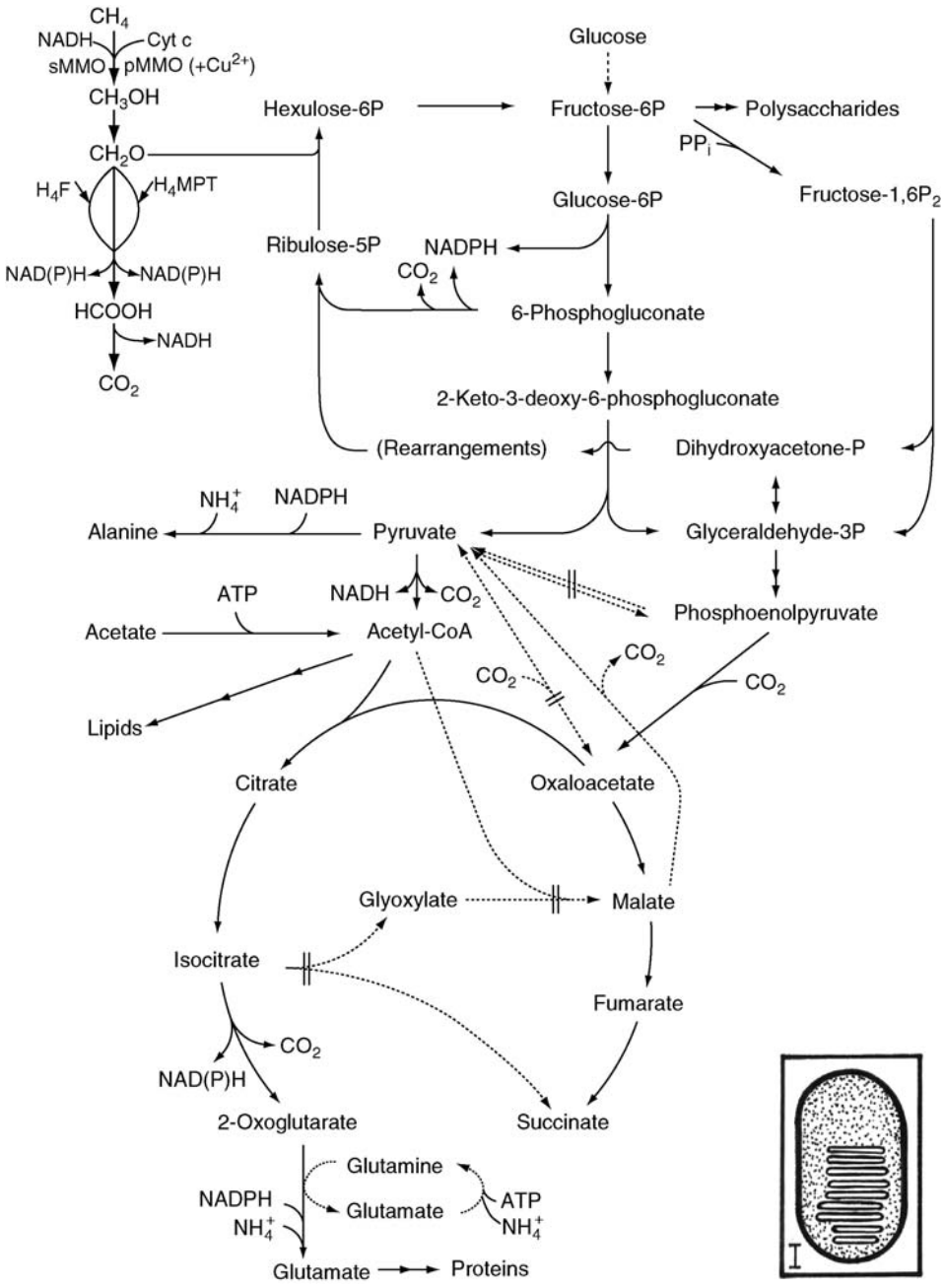

Figure 1

Pathways of carbon metabolism in Type I methanotrophs.

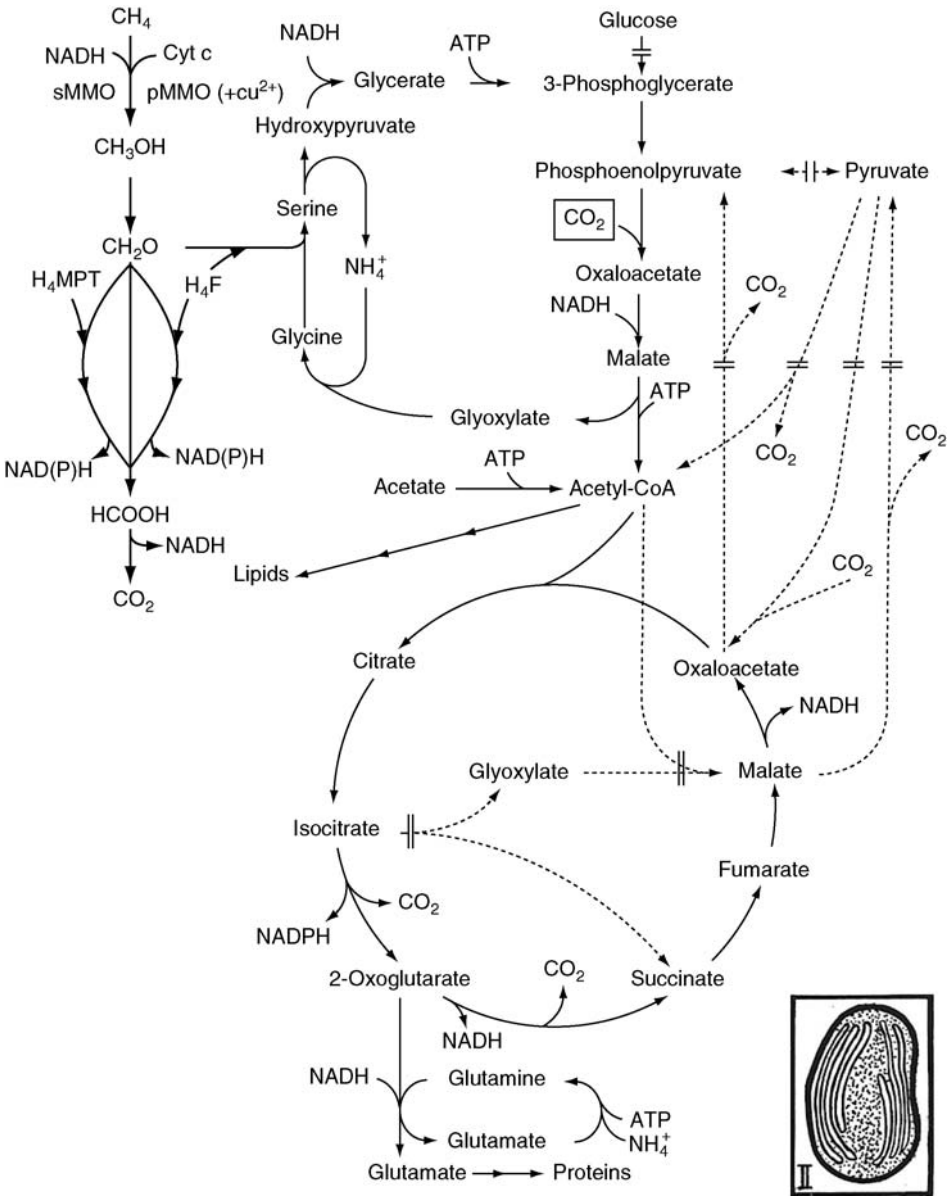

Figure 2

Pathways of carbon metabolism in Type II methanotrophs.

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). Alternatively, in the second variant fructose-6-phosphate is phosphorylated by ATP into fructose-1,6-bisphosphate, followed by aldolase cleavage to GAP and dihydroacetonephosphate, the latter being isomerized to GAP. Nonetheless, the glycolytic cleavage of phosphohexoses into phosphotrioses has not been considered as a physiologically significant pathway in methano-

trophs because of the low or zero activity of ATP-dependent 6-phosphofructokinase (Strom et al., 1974). However, the discovery of very active pyrophosphate-dependent 6-phosphofructokinase (PP_i-PFK), together with the high intracellular levels of PP_i (up to 20 mM), have established that PP_i, but not ATP, is the phosphoryl donor in this. Furthermore, the purified PFK of *Methylomonas methanica* 12, a homodimer of 2 × 45 kDa, is rather similar in kinetic and regulatory properties to the analogous enzymes from microbes rather than plants.

The recent identification and cloning of the *pfk* gene revealed its distant similarity (16.5% identical amino sequences) to that of *Mc. capsulatus* (Bath) and the facultative methylotroph *Amycolatopsis methanolica* (23.2%). This finding suggests a different origin and metabolic role for the PP_i-PFKs among these bacteria. The high degree of divergence of the *pfk* gene in methanotrophs may be determined by the characteristics of primary and central metabolism among these bacteria, as well as with differences in the function and activity of the PP_i-PFK. Significantly, PP_i-PFK having a crucial position at a metabolic cross-roads catalyzes the easily reversible interconversion of fructose-6-phosphate into fructose-1,6-bisphosphate and participates in distribution of the carbon flux between the glycolytic and KDPG segments (branches) of the RuMP cycle. In fact, this metabolic loop serves to balance the levels of GAP and (phosphoenol) pyruvate in the cell (Trotsenko and Murrell, 2008).

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

Type II methanotrophs employ the serine cycle for C_1-assimilation. In the first part of the serine cycle, formaldehyde (after condensation with THF and formation of N^5,N^{10}-methylene THF) reacts with glycine to produce serine by the action of the appropriate serine-hydroxytransmethylase (SHTM). The amino group of serine is then transferred by a specific serine-glyoxylate aminotransferase (SGAT) to glyoxylate thus forming glycine and hydroxypyruvate (by hydroxypyruvate reductase, HPR) which is phosphorylated by ATP-glycerate kinase to 2-phosphoglycerate, followed by isomerization to PEP and its subsequent carboxylation to oxaloacetate. The reduction of oxaloacetate, catalyzed 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 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. Interestingly, the obligate methanotrophs and serine pathway methylotrophs "lacked" isocitrate lyase (icl^- variant). As shown recently, glyoxylate can be regenerated via the formation of acetoacetyl-CoA and 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. However, at present it is not clear whether this rather complicated pathway proposed for *M. extorquens* AM1 or other variants of this pathway (via citramalate or methylmalate) operates in Type II methanotrophs (Meister et al., 2005).

Methanotrophs of the genera *Methylococcus* and *Methylocaldum* assimilate formaldehyde mainly by the RuMP pathway, although they also possess less active enzymes for the serine pathway and the Calvin-Benson-Basham (CBB) cycle, i.e., phosphoribulokinase and ribulosebisphosphate carboxylase/oxygenase (Rubisco). The role of these enzyme activities is still unclear. In Type I methanotrophs, 5–15% of the carbon in cell biomass is derived from CO_2 while in Type II methanotrophs it is up to 50%. Moreover, in both Type I and Type II methanotrophs, PEP carboxylase is responsible for anaplerotic CO_2 fixation (Shishkina and Trotsenko,

1982). In the Type I methanotrophs *Methylococcus* the CBB cycle enzymes are also involved in CO_2 fixation. Rubisco of *Mc. capsulatus* (Bath) has an $\alpha_6\beta_6$ structure which differs from the typical hexadecameric structure ($\alpha_8\beta_8$) of the Form I Rubisco formed in *Proteobacteria* and also in cyanobacteria and higher plants. The Rubisco genes encoding the large subunit (*cbb*L), small subunit (*cbbS*) and putative regulatory gene (*cbbQ*) are located on one cluster in *Methylococcus* (Ward et al., 2004; reviewed in Kelly et al., 2005).

7 Nitrogen Metabolism in Methanotrophs

The ability to fix N_2 by Type II methanotrophs and *Mc. capsulatus* (Bath) has been known for some time. More recently, the potential for other Type I methanotrophs such as *Methylomonas* and *Methylobacter* to fix N_2 has also been determined by screening for the gene *nif*H which encodes the Fe-containing protein of nitrogenase. The structural genes for nitrogenase of *Mc. capsulatus* (Bath) (*nifH, nifD* and *nifK*) are contiguous as they are in other diazotrophs and analysis of the genome of *Mc. capsulatus* has revealed the presence of other *nif* genes involved in synthesis and maturation of the nitrogenase iron-molybdenum cofactor.

Type I methanotrophs assimilated NH_4^+ mainly by reductive amination of pyruvate and/or α-ketoglutarate, whereas Type II methanotrophs used 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. The structural gene for GS from *Mc. capsulatus* (Bath) has been cloned and sequenced. Its nucleotide sequence has 59% similarity with *gln*A gene of *Anabaena* sp. 7120. Regulation of *gln*A in this methanotrophs is analogous to that of enterobacteria and occurs via the Ntr system. In *Mc. capsulatus* (Bath) and Type I methanotrophs grown on medium containing ammonia, the reductive amination of pyruvate (via alanine dehydrogenase) and/or α-ketoglutarate (via glutamate dehydrogenase) occurs under high ammonia growth conditions. In contrast, when grown under N_2-fixing conditions, i.e., under ammonium limitation (<0.5 mM) or on medium containing nitrate (in the absence of NH_4^+) these methanotrophs assimilate ammonia via the glutamate cycle (reviewed in Trotsenko and Murrell, 2008). Four predicted ammonium transporters have been identified in the *Mc. capsulatus* (Bath) genome. Methanotrophs also readily use nitrate as a nitrogen source and the presence of genes encoding assimilatory nitrate reductase in the *Mc. capsulatus* genome has also been confirmed (Ward et al., 2004).

8 The Obligate Nature of Methanotrophs

Methanotrophs can only use methane or methanol as sole carbon source despite being able to co-oxidize a large number of aliphatic and aromatic compounds. The only exception known to date which is well-characterized is *Methylocella silvestris*. The basis for the obligate nature of methanotrophs is not fully understood but the recent availability of the genome sequences of *Mc. capsulatus* and *M. silvestris* should aid comparative genomics. Molecular biological and biochemical studies are needed to unravel this longstanding mystery, since there are clearly some genes encoding putative membrane transport systems for organic acids and sugars in *Mc. capsulatus*.

One attractive hypothesis explaining the inability of most methanotrophs to grow on multi-carbon substrates may be due to the lack of functionally active α-ketoglutarate dehydrogenase (even though the genes coding E_1 and E_2 structural components of α-ketoglutarate dehydrogenase are present in *Mc. capsulatus*), isocitrate lyase and malate synthase, resulting in an incomplete tricarboxylic acid (TCA) cycle and a deficient glyoxylate shunt. In Type II methanotrophs with the serine pathway, α-ketoglutarate dehydrogenase is present, i.e., there is a complete set of the TCA cycle enzymes. These bacteria also have acetyl-CoA synthetase providing an initial involvement of acetate in the TCA cycle, so that, in principle, they can obtain metabolic energy and reducing equivalents from acetate. However, due to the lack of the glyoxylate bypass enzymes, isocitrate lyase and malate synthase, the formation of C_3- and C_4-intermediates from C_2-compounds is not possible. In addition, in Type II methanotrophs there is no activity of the E_1 component of pyruvate dehydrogenase complex as well as pyruvate kinase, pyruvate carboxylase, phosphoenolpyruvate (PEP) synthetase, and pyruvate phosphate dikinase which leads to a limited metabolism of pyruvate. Remarkably, the gluconeogenic pathway from C_3- and C_4-compounds (by means of pyruvate carboxylation to malate followed by its decarboxylation to PEP) is also inoperative because of the lack of pyruvate carboxylase and PEP carboxykinase in Type II methanotrophs. Moreover, the low activity of α-ketoglutarate dehydrogenase suggests an anabolic role for the TCA cycle in these bacteria (Shishkina and Trotsenko, 1982). Full annotation and comparative analysis of the genome sequences of methanotrophs which are currently being sequenced will provide a metabolic "blueprint" enabling the design of experiments to address the longstanding question of "obligate methanotrophy" and readers are referred to an extensive review on this subject by Wood et al. (2004) for further thought-provoking discussions.

9 Methanotrophs and Biotechnology

In the 1970s there was interest in the use of methanotrophs for the production of single-cell protein from the cheap and readily available substrate methane. Other cheaper sources of protein from soya curtailed these early efforts, but more recently interest in production of single-cell protein from methane and methanol has been revived in Denmark and Norway for production of added-value protein products as amino acid-balanced feed for fish and other animals. Methanotrophs have also been investigated for production of bioplastics from polyhydroxybutyrate (PHB). Halotolerant methanotrophs have also been studied for their ability to accumulate substantial amounts of the osmolyte ectoine, which can be used as a moisturizer in the cosmetic industry (Trotsenko et al., 2005). Interest in methanotrophic bacteria as biocatalysts for synthetic chemistry and bioremediation has arisen from the unique catalytic properties of the two MMO systems, most importantly their ability (1) to oxidize methane to methanol and (2) to co-oxidize a wide range of other substrates. Both systems require an exogenous source of reductant for the monooxygenation reaction, which in whole-cell applications can be supplied by adding methanol, formate, or hydrogen. sMMO has a remarkably wide substrate range, oxidizing not only methane (the natural substrate) but also co-oxidizing a large number of organic compounds. Alkanes are hydroxylated mostly at the terminal and subterminal positions. Ring hydroxylation of aromatics occurs primarily at the meta position. sMMO oxygenates alkenes to epoxides with retention of stereochemistry around the C=C double bond. A pilot process for production of epoxypropane from propane using sMMO-expressing *Mc. capsulatus* cells has been demonstrated by Dalton and colleagues. Methanol was used as the reductant and

inhibition of sMMO by the epoxide product was overcome by operating the process in a continuous two-stage system (reviewed in Smith and Murrell, 2008).

The initial oxygenated products formed from halogenated substrates may decompose rapidly via non-enzymic pathways that result in the loss of halogen substituents. The priority pollutant trichloroethylene (TCE) is a substrate for both forms of MMO and, by a combination of enzyme-catalyzed oxygenation and nonenzymatic steps, pMMO-expressing methanotroph cells can lead to TCE mineralization to CO_2, water and chloride. Several studies have used methanotrophs for bioremediation of groundwater and effluents contaminated with TCE and other chlorinated solvents. For example, a TCE-contaminated aquifer in Japan has been biostimulated with methane and inorganic nutrients to promote growth of methanotrophic bacteria to degrade the TCE (Iwamoto et al., 2000). Pilot studies have also used methanotrophs in ex situ systems for bioremediation of chlorinated organic solvents such as TCE and *cis*-1,2-dichloroethylene. Bioremediation considerations include the potential supply of reductant for MMO enzymes, often formate or methanol instead of the natural substrate methane. Manipulation of sMMO enzymes in the future may make them more effective in degrading mono- and di-aromatic pollutants (including polychlorinated biphenyls) and even polyaromatic hydrocarbons.

Advances in genetic methods for methane oxidizers have made possible the use of these organisms as hosts for production of recombinant and heterologous proteins, including β-glucuronidase and genetically engineered monooxygenases. Recently DuPont reported the engineering of *Methylomonas* sp. to produce the carotenoid astaxanthin, providing proof-of-concept for metabolic engineering of methanotrophs to synthesize new small-molecule products.

10 Research Needs

Improved genetic systems for methanotrophs such as *Ms. trichosporium* and *Mc. capsulatus*, together with a high activity homologous expression system for methanotrophs, will almost certainly 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 objective 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, for example, grow at high or low temperatures, more extreme pH values and which have solvent resistance may improve the potential use of methanotrophs in bioremediation 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 these membranes 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 of *Methylococcus capsulatus* (Bath) have been carried out; these and future genomic studies 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. Future postgenomic studies, using 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.

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