REVIEW ARTICLE

Methanotrophs and copper

Jeremy D. Semrau[1], Alan A. DiSpirito[2] & Sukhwan Yoon[1]

[1]Department of Civil and Environmental Engineering, The University of Michigan, Ann Arbor, MI, USA; and [2]Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA, USA

Correspondence: Jeremy D. Semrau, Department of Civil and Environmental Engineering, The University of Michigan, 117 EWRE Bldg, 1351 Beal Avenue, Ann Arbor, MI 48109-2125, USA. Tel.: +1 734 764 6487; fax: +1 734 763 2275; e-mail: jsemrau@umich.edu

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Abstract

Methanotrophs, cells that consume methane (CH_4) as their sole source of carbon and energy, play key roles in the global carbon cycle, including controlling anthropogenic and natural emissions of CH_4, the second-most important greenhouse gas after carbon dioxide. These cells have also been widely used for bioremediation of chlorinated solvents, and help sustain diverse microbial communities as well as higher organisms through the conversion of CH_4 to complex organic compounds (e.g. in deep ocean and subterranean environments with substantial CH_4 fluxes). It has been well-known for over 30 years that copper (Cu) plays a key role in the physiology and activity of methanotrophs, but it is only recently that we have begun to understand how these cells collect Cu, the role Cu plays in CH_4 oxidation by the particulate CH_4 monooxygenase, the effect of Cu on the proteome, and how Cu affects the ability of methanotrophs to oxidize different substrates. Here we summarize the current state of knowledge of the phylogeny, environmental distribution, and potential applications of methanotrophs for regional and global issues, as well as the role of Cu in regulating gene expression and proteome in these cells, its effects on enzymatic and whole-cell activity, and the novel Cu uptake system used by methanotrophs.

Introduction

Methanotrophs were first identified in 1906 (Söhngen, 1906) and are distinguished from other microorganisms by the ability to utilize methane (CH_4) as their sole carbon and energy source. Methanotrophs play a major role in the global cycling of carbon, nitrogen, and oxygen as well as in the degradation of hazardous organic materials. Most known methanotrophs grow best at moderate pH (5–8) and temperature ranges (20–35 °C), but psychrophilic (growth < 15 °C), thermophilic (growth > 40 °C), alkaliphilic (growth at pH > 9.0), and acidophilic (growth at pH < 5) methanotrophs have been isolated.

Despite the diversity of methanotrophs and the wide range of environments in which they are found, the general pathway by which these cells oxidize CH_4 to CO_2 is remarkably similar with methanol, formaldehyde and formate as intermediates (Fig. 1). This pathway, although relatively simple, belies a great deal of complexity as multiple enzymatic systems carry out some of these transformations, particularly in the initial oxidation of CH_4 to methanol as well as in the pathway by which carbon is assimilated into biomass.

For the conversion of CH_4 to methanol, two forms of methane monooxygenase have been found. 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. In methanotrophs that have both forms of MMO, copper (Cu) is known to be a key factor in regulating the expression of the genes encoding both sMMO and pMMO as well as the activity of these enzymes (Takeda *et al.*, 1976; Takeda & Tanaka, 1980; Scott *et al.*, 1981; Stanley *et al.*, 1983; Dalton *et al.*, 1984). For example, in *Methylosinus trichosporium* OB3B, using standard nitrate mineral salts (NMS) medium (Whittenbury *et al.*, 1970), no sMMO activity was detectable at Cu-to-biomass ratios $> 5.64\ \mu mol\ Cu\ g^{-1}$ protein (Morton *et al.*, 2000a).

The sMMO is a well characterized, three component enzyme consisting of a hydroxylase, a reductase, and a

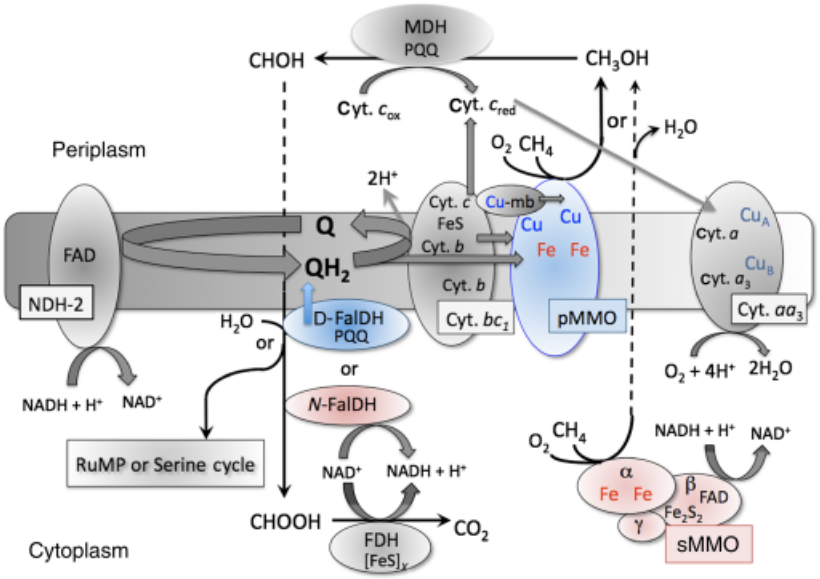

Fig. 1. Proposed pathway of CH_4 oxidation in cells cultured under high and low Cu conditions. Proteins showing positive or negative Cu-regulation are shown in blue and red, respectively. Cyt, cytochrome; D-FalDH, dye-linked/quinone-linked formaldehyde dehydrogenase; FDH, formate dehydrogenase; N-FalDH, NAD(P)-linked formaldehyde dehydrogenase; NDH-2, type 2 NADH dehydrogenase; pMMO, membrane-associated or particulate methane monooxygenase; Q, ubiquinone; FAD, flavin adenine dinucleotide; MDH, methanol dehydrogenase; PQQ, pyroloquinoline quinone; sMMO, cytoplasmic or soluble methane monooxygenase; RuMP, ribulose monophosphate.

regulatory protein (Colby *et al.*, 1977; Colby & Dalton, 1978, 1979; Woodland & Dalton, 1984; Green & Dalton, 1985; Fox *et al.*, 1989; Pilkington & Dalton, 1990; Wallar & Lipscomb, 1996, 2001). The hydroxylase component is composed of three subunits with molecular masses of approximately 54 000 Da (α-subunit), 42 000 Da (β-subunit), and 22 000 Da (γ-subunit) with a subunit molecular structure of $(\alpha\beta\gamma)_2$. Spectroscopic and X-ray crystallographic studies have firmly established that the 54 000 Da polypeptide of the hydroxylase contains an oxygen-bridged diiron cluster as the site of CH_4 catalysis (Fox *et al.*, 1988, 1989; Rosenzweig *et al.*, 1993; Elango *et al.*, 1997). Similar iron centers have been found in a variety of proteins, including ribonucleotide reductase and stearoyl-ACPΔ^9 desaturase (Merkx *et al.*, 2001; Kolberg *et al.*, 2004). The reductase component is NADH-dependent and composed of one polypeptide with a molecular mass of 38–40 000 Da containing both FAD and Fe-S cofactors (Fox *et al.*, 1989). Component B is a 15–17 000 Da regulatory protein (Green & Dalton, 1985; Wallar & Lipscomb, 2001). The reader is referred to several excellent reviews for additional information on the sMMO (Lipscomb, 1994; Wallar & Lipscomb, 1996; Walters *et al.*, 1999; Murrell *et al.*, 2000; Dalton, 2005). With respect to this review, in addition to repression of expression, sMMO activity is inhibited by Cu in both whole-cell and cell-free fractions (Takeda *et al.*, 1976; Dalton *et al.*, 1984).

In contrast to the sMMO, little is known about the molecular properties of the pMMO, mainly due to the low specific activity of most enzyme preparations (Zahn & DiSpirito, 1996;Nguyen *et al.*, 1998; Takeguchi *et al.*, 1998; Basu *et al.*, 2003; Lieberman *et al.*, 2003; Choi *et al.*, 2005). Laboratories studying the pMMO agree that the pMMO is a Cu-containing enzyme, composed of three polypeptides with molecular masses of approximately 45 000 Da (α-subunit, PmoB), 26 000 Da (β-subunit, PmoA), and 23 000 Da (γ-subunit, PmoC), with a $(\alpha\beta\gamma)_3$ subunit structure (Zahn & DiSpirito, 1996; Nguyen *et al.*, 1998; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman & Rosenzweig 2005). However, researchers in the field disagree on the number, type, and function of metal centers associated with the pMMO, as well as the nature of the physiological electron donor.

In this review, we summarize information collected to date on the phylogeny, distribution, environmental roles, application, and enzymology of methanotrophs, with particular focus on the role of Cu in methanotrophic physiology and different models are presented for the metal centers of pMMO.

Methanotrophic phylogeny and taxonomy

Although methanotrophs were first identified as early as 1906, it was not until the 1970s that extensive isolation and characterization of these cells was performed, allowing for detailed phylogenetic and physiological analyses of these cells (Whittenbury *et al.*, 1970). Initially, these cells were grouped into three 'Types,' i.e., Type I, II, and X. Type I strains were characterized by having, among other characteristics: (1) intracytoplasmic membranes throughout the cell as bundles of vesicular disks; (2) utilization of the ribulose monophosphate (RuMP) pathway for carbon assimilation; and (3) signature phospholipid fatty acids of 14 and 16 carbons in length. Type II strains were characterized as having: (1) intracytoplasmic membranes aligned along the periphery of the cell; (2) utilization of the serine pathway

for carbon assimilation; and (3) signature phospholipid fatty acids 18 carbons in length. Type X strains on the other hand, had characteristics of both types, including 16 carbon phospholipid fatty acids, the RuMP pathway as well as possessing ribulose-1,5-bisphosphate, and typically growing at higher temperatures than Type I or II strains (Hanson & Hanson, 1996). Subsequently, others have determined using a combination of biochemical and molecular analyses that *Proteobacteria* methanotrophs should be grouped as either Type I or II, i.e., Type X strains are now reclassified as being a subset of Type I methanotrophs (Bowman *et al.*, 1993).

In the classic review of Hanson & Hanson (1996), methanotrophs were grouped into six genera in the *Proteobacteria*. Based on 16S rRNA gene sequence analyses Type I methanotrophs grouped in the *Gammaproteobacteria* either as *Methylobacter*, *Methylococcus*, *Methylomicrobium*, and *Methylomonas*, while Type II methanotrophs grouped in the *Alphaproteobacteria* as either *Methylocystis* or *Methylosinus* strains. Almost 15 years later, 16 genera have now been described within *Proteobacteria*, with 12 in the *Gammaproteobacteria* and four in the *Alphaproteobacteria*. New genera within the *Gammaproteobacteria* include *Methylocaldum*, *Methylohalobius*, *Methtyhlothermus*, *Methylosarcina*, *Methylosoma*, and *Methylosphaera* (Bodrossy *et al.*, 1997, 1999; Bowman *et al.*, 1997; Wise *et al.*, 2001; Heyer *et al.*, 2005; Kalyuzhnaya *et al.*, 2005; Rahalkar *et al.*, 2007). Furthermore, more 'unusual' filamentous methanotrophs have been discovered within the genera *Clonothrix* and *Crenothrix* (Stoecker *et al.*, 2006; Vigliotta *et al.*, 2007), but these are also considered to be Type I methanotrophs as they and the other genera are phylogenetic subsets of the *Methylococcaceae* family (Op den Camp *et al.*, 2009). A summary of *Gammaproteobacteria* genera and their characteristics are shown in Table 1 (with the exception of *Crenothrix* and *Clonothrix* due to the current inability to isolate and cultivate these cells in pure cultures).

New genera within the *Alphaproteobacteria* include *Methylocella* and *Methylocapsa*, strains isolated from sphangnum peat bogs and acidic forest soils (Dedysh *et al.*, 2000, 2002, 2004; Dunfield *et al.*, 2003). These cells utilize the serine pathway for carbon assimilation, but are not considered to be classic Type II methanotrophs as they are within the *Beijierinckiacaea* family, and not *Methylocystaceae* as *Methylosinus* and *Methylocystis* are. Furthermore, these cells either exhibit unique intracytoplasmic membrane arrangements, or do not have any intracytoplasmic membranes, and also are moderately acidophilic (growth at pH as low as 4.2). The characteristics of *Alphaproteobacteria* methanotrophs are shown in Table 2.

Known methanotrophic diversity increased dramatically with three simultaneous and independent reports of the isolation and characterization of methanotrophs grouping not within the phylum *Proteobacteria*, but within *Verrucomicrobia* (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). Relatively little is known about *Verrucomicrobia* in general, although they may constitute as much as 10% of all soil bacteria and 10–12% of all bacterial 16S rRNA in soils (Buckley & Schmidt, 2002; Sangwan *et al.*, 2005; Wagner & Horn, 2006), and the finding of *Verrucomicrobia* methanotrophs indicates that these cells may play significant roles in global carbon cycling, but yet of unknown magnitude. These cells, although isolated from geothermal locations quite distant from each other [New Zealand, Kamchatka (Russia), and Southern Italy], have remarkable 16S sequence similarity (>98%) and are proposed to be representatives of the genus *Methylacidiphilum* (Op den Camp *et al.*, 2009). These strains lack any intracytoplasmic membranes, but genomic analyses indicated a complete Calvin–Benson–Bassham cycle, some evidence of carboxysome-like structures were observed in TEM images, and CO_2 stimulated growth of these cells (Dunfield *et al.*, 2007; Pol *et al.*, 2007). A summary of the characteristics of *Verrucomicrobia* methanotrophs is shown in Table 3, with a comparison of the currently identified general groups of methanotrophs provided in Table 4.

The current phylogenetic distribution of known methanotrophic genera based on 16S rRNA gene sequences is shown in Fig. 2. Clearly a wide phylogenetic distribution exists, with three general groups in the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia*. A broad diversity within the *Gammaproteobacteria* (*Crenothrix*, *Clonothrix*, *Methylomonas*, *Methylobacter*, *Methylosarcina*, *Methylococcus*, *Methylocaldum*, and *Methylothermus* and *Methylomicrobium*) is clear, and this is reflected in the broad environmental conditions they are found (see following section on the environmental diversity of methanotrophs). The *Alphaproteobacteria* currently include *Methylosinus*, *Methylocystis Methylocapsa*, and *Methylocella*, while the *Verrucomicrobia* are represented by *Methyloacidiphilum* strains.

Phylogenetic relationships between methanotrophs are also commonly examined considering the sequences of *pmoA*, encoding for the β-subunit of the pMMO. With the exception of the genus *Methylocella*, all methanotrophs have the structural genes for the pMMO (Theisen *et al.*, 2005), and most known methanotrophs have multiple copies of the *pmo* operon. As shown in Fig. 3, the unusual *pmoA* sequence of *Crenothrix* diverges from other *Gammaproteobacteria* methanotrophs, as well as that the sequence of one copy of *pmoA* in the *Verrucomicrobia* strains is very divergent from other sequences found in the same cell. These findings indicate that the presence of *pmo* genes in *Methylacidophilum* and *Crenothrix* was not due to a recent horizontal gene transfer event, but rather that these cells diverged from other methanotrophs some time ago. The significant sequence divergence of *pmoA* also suggests that other methanotrophs

Table 1. Characteristics of methanotrophic genera within *Gammaproteobacteria*

Characteristic	*Methylobacter*	*Methylococcus*	*Methylocaldum*	*Methylohalobius*	*Methylomicrobium*	*Methylomonas*	*Methylosoma*	*Methylosphaera*	*Methylosarcina*	*Methylothermus*
Cell morphology	Cocci-ellipses	Cocci-rods	Cocci-rods	Cocci	Rods	Rods	Cocci-rods	Cocci-rods	Cocci, rods, coccobacillary, or fusiform	Cocci
Motility	V*	−	+	+	+	+	−	−		−
Cyst formation	+	+	+	−	−	+	+	−	+	−
Desiccation resistance	+	−	V*	−	−	−	−	−	−	−
sMMO	−	+	−	−	−	−	−	−	−	−
pMMO	+	+	+	+	+	+	+	+	+	+
N fixation	−	+	−	−	−	V*	+	+	−	−
Rubisco	−	+	+	−	−	−	NR	−	−	−
pH growth range	5.5–9.5	NR	6–8.5	6.5–7.5	6–9	5.5–8.5	5–9	NR	4–9	6.5–7.5
Temperature growth range (°C)	0–40	25–65	20–62	15–42	10–30	10–42	16–30	0–21	4–35	37–72
Salt concentration range (% NaCl)	∼0.3–4	NR	0.1–0.5	1.0–15	∼0.3–12	NR	< 0.5	∼1.8–3.5†	∼1	0.5–1.0
Major PLFAs	16:1ω7c; 16:1ω5t	16:0; 16:1ω7c	16:0; 16:1ω7c	18:1ω7; 16:0; 16:1ω7	16:1ω5t; 16:1ω7c	16:1ω8c; 14:0	16:1ω7c	16:1ω8c; 16:1ω7c; 16:1ω6c; 16:0	16:1ω7c; 16:1ω5t; 16:0	16:0; 18:1ωc
G+C (mol%)	48–55	62–65	57–59	59	48–60	50–59	49.9	43–46	53–54	62.5

*Varies between species.

†Requires sea water for growth.

References: Sieburth *et al*. (1987), Bodrossy *et al*. (1997), Bowman *et al*. (1997), Wise *et al*. (2001), Trotsenko & Khmelenina (2002), Heyer *et al*. (2005), Tsubota *et al*. (2005), Kalyuzhnaya *et al*. (2005), Bowman (2006), and Rahalkar *et al*. (2007).

NR, not reported.

Table 2. Characteristics of methanotrophic genera within *Alphaproteobacteria*

Characteristic	*Methylocystis*	*Methylosinus*	*Methylocapsa*	*Methylocella*
Family	*Methylocystaceae*	*Methylocystaceae*	*Beijerinckaceae*	*Beijerinckaceae*
Cell morphology	Pyriform or vibroid	Reniform to rodlike	Curved cocci	Bipolar straight or curved rods
Intracytoplasmic membrane arrangement	Parallel to cell periphery	Parallel to cell periphery	Membrane vesicles parallel to long axis on one side of cell membrane	Vesicular membranes connected to cytoplasmic membrane
Cyst formation	Varies between species	−	+	−
Exospore formation	−	+	−	+
Motility	−	+	−	−
pMMO	+	+	+	−
sMMO	Varies between species	+	−	+
Growth at pH 5	−	−	+	+
Growth at 0.5% salt	+	+	−	−
Major PLFAs	18:1ω8c	18:1ω8c	18:1ω7c	18:1ω7c
G+C content (mol%)	62–67	62–67	61	60–63

References: Dedysh *et al*. (2000, 2002, 2004), Dunfield *et al*. (2003), and Bowman (2006).

Table 3. General characteristics of *Verrucomicrobia* methanotrophs

Characteristic	
Motility	−
Cyst formation	−
sMMO	−
pMMO	+
Nitrogen fixation	+
RuMP pathway	−
Serine pathway	+
Rubisco	+
Intracytoplasmic membrane formation	Vesicular membranes
Carboxysome-like structures	+
Temperature growth range (°C)	37–65
pH growth range	0.8–6.0
Major PLFAs	I14 : 0, a15 : 0, 18 : 0
G+C (mol%)	40.8–45.5
Facultative	−

References: Dunfield *et al*. (2007), Pol *et al*. (2007), Islam *et al*. (2008), Op den Camp *et al*. (2009).

within the phyla of *Proteobacteria* and *Verrucomicrobia*, as well as in other phyla, may exist.

Methanotrophic environmental diversity

Thermotolerant and thermophilic methanotrophs

It has been known for quite some time that methanotrophs are fairly ubiquitous in environmental samples, being found, among other places, wetlands, freshwater and marine sediments and water columns, sewage sludge, groundwater, rice paddies, and peat bogs (Bowman, 2006 and references therein; Hanson & Hanson, 1996 and references therein; Dedysh *et al*., 1998b and references therein). Early work characterizing the distribution of methanotrophs found that most methanotrophs were neutrophilic and mesophilic, i.e., optimal growth at neutral or near-neutral pH, and moderate temperature (~25 °C), although *Methylococcus* strains, for example, *Methylococcus capsulatus* Bath, have an optimal growth temperature of 45 °C.

More recently another genus within the *Gammaproteobacteria*, *Methylocaldum*, has also been found that has both thermotolerant and thermophilic species. *Methylocaldum tepidum* and *Methylocaldum gracile* are thermotolerant, growing between 30–47 and 20–47 °C, respectively, with both having optimal growth at 42 °C. *Methylocaldum szegendiense* is thermophilic, with growth occurring between 37 and 62 °C, and optimal growth at 55 °C (Bodrossy *et al*., 1997). An additional genus of thermophilic methanotrophs has also been identified, *Methylothermus*. This genus was first proposed with the isolation of strain HB from a hot spring in Hungary, and was reported to grow between 40 and 72 °C, with optimal growth between 62 and 65 °C (Bodrossy *et al*., 1999). This strain, however, was not extensively characterized and is no longer extant (Tsubota *et al*., 2005). Its finding, however, led to the isolation and characterization of *Methylothermus thermalis* for a hot spring in Japan, validating the *Methylothermus* genus. This cell is able to grow between 37 and 67 °C, with optimal growth between 57 and 59 °C. It is interesting to note that *M. thermalis*, although grouping with the *Gammaproteobacteria*, has high abundances of both 16 and 18 carbon fatty acids, i.e., signatures of both Type I and II methanotrophs (Tsubota *et al*., 2005).

Table 4. General characteristics of known families of methanotrophs

Characteristic				
Phylum	*Gammaproteobacteria*	*Alphaproteobacteria*	*Alphaproteobacteria*	*Verrucomicrobia*
Family	*Methylococcaceae*	*Methylocystaceae*	*Beijerinckaceae*	*Methylacidiphilaceae*
Genera	*Methylobacter, Methylococcus, Methylocaldum, Methylohalobius, Methylomicrobium, Methylomonas, Methylosoma, Methylosarcina, Methylosphaera, Methylothermus, Crenothrix, Clonothrix*	*Methylosinus, Methylocystis*	*Methylocapsa, Methylocella*	*Methylacidiphilum*
RuMP pathway	+	−	−	−
Serine pathway	−	+	+	+
Rubisco	Rarely	−	−	+
sMMO	Varies between species	Varies between species	Varies between species	−
pMMO	+	+	Varies between species	+
Nitrogen fixation	Varies between species	Varies between species	+	Varies between species
Intracytoplasmic membrane formation	Bundles of disks perpendicular to cell periphery	Membrane stacks parallel to cell periphery	*Methylocapsa* – membrane vesicles parallel to long axis on one side of cell membrane. *Methylocella* – cytoplasmic membrane invaginations	−
Carboxysome-like structures or vesicles	−	−	+	+
Major PLFAs	14:0; 16 : 0; 16:1ω5t; 16:1ω6c; 16:1ω7; 16:1ω7c; 16:1ω8c; 18:1ω7	18:1ω7c; 18:1ω8c	18:1ω7c	i14:0; a15:0; 18:0
Resting stages	Varies – cysts or none	Varies – cysts, spores or none	Cysts or spores	None
G+C mol%	43–65	62–67	60–63	41–46
Facultative	−	−	Varies between species	−

References: Sieburth *et al*. (1987), Bodrossy *et al*. (1997), Bowman *et al*. (1997), Wise *et al*. (2001), Dedysh *et al*. (2002), Trotsenko & Khmelenina (2002), Dunfield *et al*. (2003, 2007), Heyer *et al*. (2005), Kalyuzhnaya *et al*. (2005), Tsubota *et al*. (2005), Bowman (2006), Pol *et al*. (2007), Rahalkar *et al*. (2007), Islam *et al*. (2008), and Op den Camp *et al*. (2009).

The *Verrucomicrobia* methanotrophs reported to date are also thermophilic, with all three isolated strains having optimal growth temperatures of 55 °C or above, and can grow at temperatures as high at 65 °C. These cells, which are also acidophilic (see below for more discussion) were isolated from: (1) mud and mixed soil samples near volcanic mudpots in Southern Italy (Pol *et al*., 2007); (2) a geothermal active area, Hell's Gate in New Zealand (Dunfield *et al*., 2007); and (3) clay and spring water samples from an acidic hot spring in Kamchatka, Russia (Islam *et al*., 2008). Provisionally, these were named *Acidomethylosilex fumaricum* SolV, *Methylokorus infernorum* strain V4, and *Methyloacida kamchatkenesis* strain Kam1, but as these cells have significant phylogenetic and physiological similarities, it has been proposed that all three cells be considered part of the new genus *Methylacidiphulum* (Op den Camp *et al*., 2009).

Less is known about the pathway of CH_4 oxidation by these *Verrucomicrobia* methanotrophs given their recent discovery. Reconstruction of carbon assimilation pathways in *Methylacidiphilum infernorum* indicates that two key enzymes of the RuMP pathway, hexulose-6-phosphate and hexulose-phosphate isomerase, are not present. 6-Phosphogluconate dehydrogenase and phospho-3-keto-3-deoxygluconate aldolase, are also absent, indicating that this cell does not utilize the RuMP pathway for assimilation of carbon from formaldehyde. Interestingly, all but two enzymes of the serine pathway were identified from the genome of *Methylacidiphilum infernorum*, i.e., malyl coenzyme A lyase and glycerate kinase, necessary for the regeneration of glyoxylate and conversion of glycerate to 3-phospoglycerate, respectively, are absent (Hou *et al*., 2008). It is suggested that glyoxylate may be formed via either the Calvin–Benson–-Bassham pathway as key genes for this pathway are present, or the glyoxylate shunt enzymes isocitrate lyase and malate synthetase (Hou *et al*., 2008; Op den Camp *et al*., 2009). It is still unknown how or if 3-phospoglycerate is formed from glycerate. It may be that carbon is actually fixed via the CBB pathway, and it has been found addition of CO_2

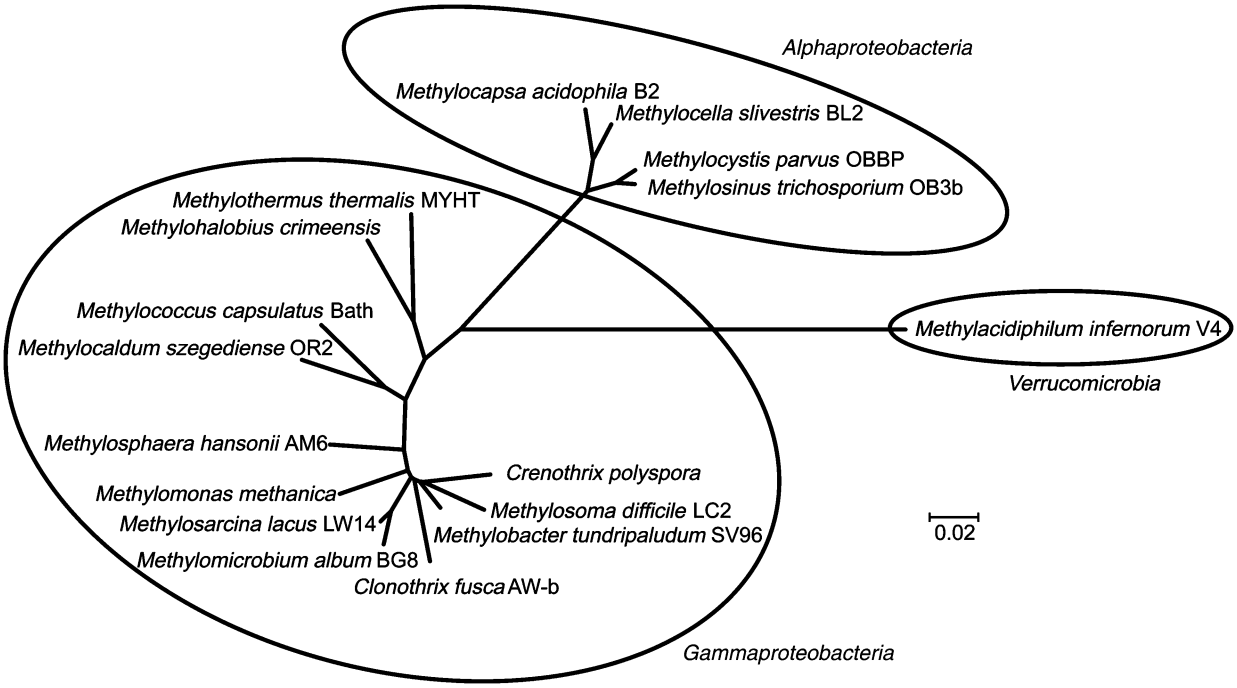

Fig. 2. Phylogenetic relationships between known methanotrophs based on 16s rRNA gene sequences using MEGA4 (Tamura *et al.*, 2007). The tree was constructed using the neighbor-joining method with 1304 positions of 16s rRNA gene. The bootstrap consensus tree was inferred from 500 replicates. Evolutionary distances were computed using the maximum composite likelihood method. The scale bar indicates 0.02 base substitutions per site.

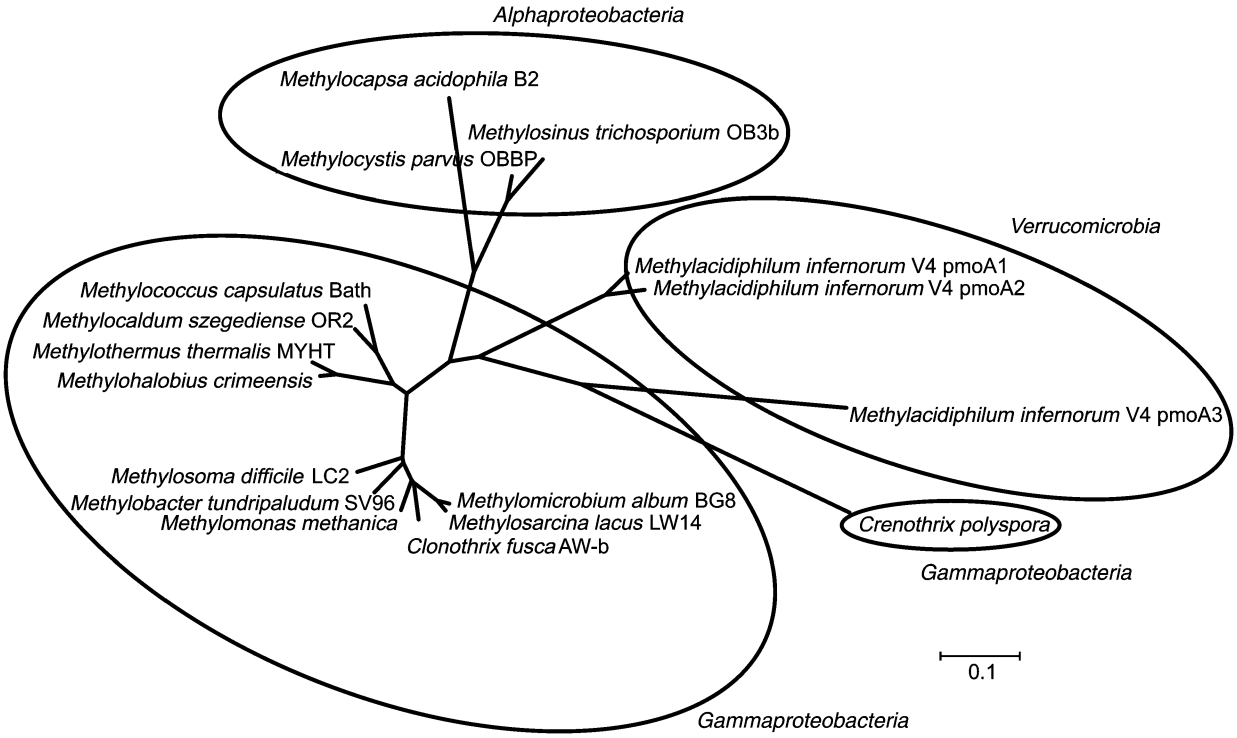

Fig. 3. Phylogenetic relationships between known methanotrophs based on deduced PmoA sequences using MEGA4 (Tamura *et al.*, 2007). The tree was constructed using the neighbor-joining method with 101 amino-acid positions. The bootstrap consensus tree was inferred from 500 replicates. Evolutionary distances were computed using the PAM Dayhoff matrix. The scale bar indicates 0.1 amino-acid substitutions per site.

substantially enhances growth of these cells (Dunfield *et al.*, 2007; Op den Camp *et al.*, 2009).

Psychrotolerant and psychrophilic methanotrophs

At the other end of the spectrum, evidence of methanotrophic activity has been found at temperatures below 10 °C (Omelchenko *et al.*, 1993; Berestovskaya *et al.*, 2002). As over 80% of the biosphere is cold, i.e., never warming above 15 °C (Russell, 1990), and many of these environments have substantial CH_4 fluxes, for example, polar tundra regions, the finding of methanotrophic activity in cold regions is not surprising. To date, however, only three psychrophilic methanotrophs have been isolated, i.e., cells with optimal growth at or below 15 °C, and these cells are all within the *Gammaproteobacteria*. The first, *Methylobacter psychrophilus*, was isolated from Russian tundra soil and grows optimally between 3.5 and 10 °C (Omel'chenko *et al.*, 1996; Tourova, 1999). Subsequently, *Methylosphaera hansonii* was isolated from salty Antarctic meromictic lakes with an optimal growth temperature between 10 and 13 °C (Bowman *et al.*, 1997). Finally, *Methylomonas scandinavica*, with an optimal growth temperature of 15 °C, was isolated from deep ($>$ 400 m) igneous ground water of Sweden (Kalyuzhnaya *et al.*, 1999).

Halotolerant/philic and alkalitolerant/philic methanotrophs

Early studies isolated *Gammaproteobacteria* methanotrophs originally assigned to the genus *Methylomonas* from marine environments, documenting the presence of halotolerant methanotrophs (Sieburth *et al.*, 1987, 1993; Lidstrom, 1988; Lees *et al.*, 1991). These have now been reclassified as *Methylomicrobium* species (Bowman *et al.*, 1995). Subsequently, molecular evidence indicated that in marine environments, *Gammaproteobacteria* methanotrophs appeared to dominate over *Alphaproteobacteria* strains as *Gammaproteobacteria* methanotrophs were easily enriched (Holmes *et al.*, 1995). Since then, methanotrophs isolated from marine waters, estuaries, arctic soil, groundwater, and soda lakes that are halotolerant, i.e., capable of growth at salt concentrations between 0.15% and 4%. These are predominantly genera within *Gammaproteobacteria*, specifically *Methylomicrobium* and *Methylobacter* (Sieburth *et al.*, 1987; Bowman *et al.*, 1993; Khmelenina *et al.*, 1997, 1999; Smith *et al.*, 1997; Fuse *et al.*, 1998; Kaluzhnaya *et al.*, 2001, 2008; Trotsenko & Khmelenina, 2002; Wartiainen *et al.*, 2006). Some *Gammaproteobacteria* methanotrophs are truly halophilic, for example, optimal growth at salt concentrations $>$ 3%. *Methylohalobius crimeensis*, isolated from hypersaline lakes (salt concentrations ranging between 23% and 26%) has optimal growth at salt concentrations of ~6–9% and pH between 6.5 and 7.5 (Heyer *et al.*, 2005). A second methanotroph, *M. hansonii*, isolated from Antarctic meromictic lakes with marine salinity, grows best in NMS medium amended with sea water (~3.5% salt) (Bowman *et al.*, 1997). *Methylomicrobium* and *Methylobacter* species isolated from soda lakes have also been found to be both halophilic and alkaliphilic methanotrophs, i.e., optimal growth occurs at pH 9.0 and at salt concentrations of ~3% or greater (Khmelenina *et al.*, 1997; Sorokin *et al.*, 2000).

An alkalitolerant *Alphaproteobacteria* strain closely related to *Methylocystis rosea* and *Methylocystis hirsuta* has also recently been isolated from Maloe Guzhirnoe soda lake (Siberia; pH = 9.7, low salt concentration). The isolate was able to grow between pH 6.0 and 9.5, with optimal growth at 7.4. (Eshinimaev *et al.*, 2008). Other halo/alkalitolerant or halo/alkaliphilic *Alphaproteobacteria* methanotrophs may exist; molecular evidence indicates the presence of these methanotrophs in Mono Lake (USA; pH = 9.8; ~8.5–9.5% salinity), Lake Suduntuiski (Siberia; pH = 9.7; 0% salinity), Lake Gorbunka (Siberia; pH = 9.5; 4% salinity), and Lake Khuzhirta (Siberia; pH = 10.2; 0% salinity) (Lin *et al.*, 2004, 2005).

Acidophilic methanotrophs

Evidence of acidophilic methanotrophs, i.e., cells with optimal growth at pH of 5.5 or less, was first indicated with the successful enrichment of methanotrophic communities from acidic ombrotrophic peat bogs by Dedysh *et al.* (1998b). In this initial study, methanotrophic enrichments had maximal CH_4 uptake at pH between 4.5 and 5.5, and highest specific growth rates were observed at pH values between 5.0 and 5.7, with a calculated maximum at 5.25. Further purification, isolation, and characterization of three strains from these acidic bogs indicated that these methanotrophs, grouping within the *Alphaproteobacteria* were closely affiliated with the acidophilic heterotrophic bacterium *Beijerinckia indica* (Dedysh *et al.*, 1998a), and that these cells comprise a new genera and species, *Methylocella palustris* with an optimal growth pH between 5.0 and 5.5 (Dedysh *et al.*, 2000). Further characterization of other *Methylocella* species isolated from acidic *Sphagnum* peat bogs and acidic forest cambisols found that these cells were also acidophiles, with optimal growth pH values of 5.5–6 for *Methylocella tundrae* (Dedysh *et al.*, 2004), and 5.5 for *Methylocella silvestris* (Dunfield *et al.*, 2003). Attempts to isolate genes for the pMMO from all these strains has been unsuccessful, and it appears that members of this genus only possess sMMO, the only known methanotrophs not to express pMMO. Not all acidophilic methanotrophs, however, are unable to express pMMO. One isolate from a *Sphagnum* peat bog, although phylogenetically closely related to *Methylocella*, had a DNA : DNA hybridization with *M. palustris* of

only 7%, indicating that this constituted a new genera and species, *Methylocapsa acidophila* (Dedysh *et al.*, 2002). This cell had an optimal growth pH of 5.0–5.5, and possesses pMMO, but cannot express sMMO. Members of both *Methylocella* and *Methylocapsa* genera are sensitive to salt concentrations, and grow best at low salt contents (Dedysh *et al.*, 2000, 2002, 2004; Dunfield *et al.*, 2003; Kolesnikov *et al.*, 2004), possibly due to greater metal bioavailability at lower pH values.

As mentioned above, thermoacidophilic methanotrophs of the *Verrucomicrobia* phylum have recently been isolated from geothermally active areas. These strains, able to grow as a pH value as low as 0.8 (Pol *et al.*, 2007), have optimal pH values ranging from 2 to 2.5 (Dunfield *et al.*, 2007), 3.5 (Islam *et al.*, 2008), and 2.0 to 5.8 (Pol *et al.*, 2007). These cells also require low salt concentrations for successful isolation, and are very sensitive to chlorine (Dunfield *et al.*, 2007).

Facultative methanotrophy

Despite the broad phylogenetic diversity of methanotrophs, and the large range of environmental conditions from which they have been isolated, most characterized cells are obligate methanotrophs, i.e., they can only grow on one-carbon compounds such as CH_4 or methanol. As described above, it has been discovered that other compounds such as carbon dioxide can enhance the growth of *Verrucomicrobia* methanotrophs, and it has also been shown that chloromethane can enhance the growth of *Methylomicrobium album* BG8 when this cell is grown on methanol (Han & Semrau, 2000). In neither case, however, can CO_2 or chloromethane serve as the sole carbon source (or energy source in the case of chloromethane). Early studies suggested that some methanotrophs may be facultative, i.e., able to utilize compounds with carbon–carbon bonds as sole growth substrates, but these were later found to either be cocultures or the findings could not be substantiated by other laboratories. For an excellent summary of the history of claims of facultative methanotrophy, the reader is directed to Theisen & Murrell (2005).

Facultative methanotrophy, however, does occur, as clearly identified by Dedysh *et al.* (2005) using a suite of experimental measurements that show *Methylocella* species, particularly *M. silvestris*, can indeed use a variety of organic acids (acetate, pyruvate, succinate, and malate) as well as ethanol as the sole growth substrate. Strain purity was verified using phase-contrast microscopy, whole-cell hybridization of both strain- and genus-specific probes for cells grown on succinate, acetate, and CH_4, as well as sequencing 50 16S rRNA gene clones each from cells grown on CH_4 or acetate. Furthermore, quantitative real-time PCR of *mmoX* showed parallel increases in the amount of *mmoX* copies and cell numbers, indicating that no contaminating cells were present. Interestingly, *M. silvestris* grew faster and more efficiently on acetate than CH_4, and that acetate inhibited CH_4 oxidation upon addition (Dedysh *et al.*, 2005). Reverse transcriptase (RT-PCR) analyses also showed that acetate repressed expression of both *mmoX* (encoding for the α-subunit of the hydroxylase component of sMMO) and *mmoR* (encoding for a σ^N-dependent transcriptional activator or enhancer-binding protein) in *M. silvestris*, and the sMMO polypeptides were absent in acetate-grown cells, regardless if CH_4 was present or not (Theisen *et al.*, 2005).

At this time it is still unclear how this cell assimilates acetate into biomass as a key enzyme of the glyoxylate cycle, isocitrate lyase is missing (Dunfield *et al.*, 2003). As suggested by Theisen *et al.* (2005), alternative pathways may be used that involve coenzyme A that have been found in *Methylobacterium extorquens* AM1, *Rhodobacter capsulatus, Rhodobacter sphaeroides*, and *Rhodispirullum rubrum* (Berg *et al.*, 2002; Korotkova *et al.*, 2002, 2005; Filatova *et al.*, 2005; Meister *et al.*, 2005), for example, the ethylmalonyl-CoA pathway that has recently been completely described (Alber *et al.*, 2006; Erb *et al.*, 2007, 2008, 2009). Finally, the finding of facultative methanotrophs suggests that multicarbon compounds may be used to support methanotrophic growth *in situ*, (Dedysh *et al.*, 2005), and can have significant implications in our understanding of the CH_4 cycle, particularly in peat bogs where acetate can be a terminal end product of metabolism, and concentrations can reach as high as 1 mM (Hines *et al.*, 2001, 2008; Duddleston *et al.*, 2002).

Finally, it has been shown that the novel filamentous methanotroph *Crenothrix polyspora* can take up acetate, and to a lesser extent, glucose, in the absence of CH_4, suggesting that this cell may be a facultative methanotroph as well (Stoecker *et al.*, 2006). Interestingly, another recently discovered filamentous methanotroph, *Clonothrix fusca*, with a close phylogenetic relationship *C. polyspora* based on 16S rRNA gene sequence, but not on *pmoA* sequence (see Figs 2 and 3), was not found to be able to grow on glucose (Vigliotta *et al.*, 2007). Future work should examine more closely if these cells are truly facultative, for example, attempts to grow *C. fusca* on acetate have not been reported, and if so, how these cells assimilate carbon from both CH_4 and acetate. Difficulties in isolating and cultivating these strains in pure culture, however, have limited research in this area.

Methanotrophic association with other organisms

Methanotrophs have been found to serve as the food base of different ecosystems, for example, they have been found as endosymbionts of mussels situated around cold seeps and hydrothermal vents in the deep ocean (Childress *et al.*, 1986; Cavanaugh *et al.*, 1987, 1992; Fisher *et al.*, 1987; Robinson *et al.*, 1998; Barry *et al.*, 2002; DeChaine *et al.*, 2006;

Duperron *et al.*, 2006). Here the methanotrophs are localized in the mussel gill tissue and likely serve as an additional, internal nutritional source for the mussels. Methanotrophs have also been found in association with sponges, snails, and tubeworms (Petersen & Dubilier, 2009 and references therein; Schmaljohann & Flügel, 1987; Schmaljohann *et al.*, 1990; Schmaljohann, 1991; Vacelet *et al.*, 1995, 1996). In all these cases, the methanotrophic symbionts appear to be *Gammaproteobacteria* methanotrophs based on 16S rRNA gene sequences (Petersen & Dubilier, 2009) although symbiotic relationships between cells closely related to the genera *Methylocella* and *Methylocapsa* with *Sphagnum* mosses have been found. Here, the methanotrophs have been found to provide ~10–15% of carbon for these nonvascular plants (Raghoebarsing *et al.*, 2005). For more information on methanotrophic symbioses, particularly with marine invertebrates, the reader is directed to the recent review by Petersen & Dubilier (2009).

Methanotrophs have been implicated in serving an important role in sustaining closed, subterranean systems. Movile Cave (Hungary), a natural subterranean passage isolated from the surface until an artificial shaft was dug in 1986, was found with localized atmospheres, 'air bells,' with appreciable CH_4 (1–2%) and oxygen concentrations (7–10%). Stable isotope analyses of microcosms composed of aqueous water samples and microbial mats collected from the cave indicated that methanotrophs were active and numerous, and that these cells apparently serve to provide substrates for not only other bacteria (e.g. methanol for *Methylophilus* and *Hyphomicrobium* species, general cell mass for bacteriovires such as *Bdellovibrio*), but also eukaryotes (e.g. *Ochromonas* also apparently acting as a bacteriovore) (Hutchens *et al.*, 2004).

Lastly, chironomid larvae have been found to graze on both *Gamma-* and *Alphaproteobacteria* methanotrophs, indicating that methanotrophs can supply a large amount of the carbon and energy required for macroinverterbrates in surface freshwater systems (Deines *et al.*, 2007). As such, it appears that methanotrophs can be a significant part of the food base for significantly different types of ecosystems.

Application of methanotrophs

Pollutant degradation

Many monooxygenases, including the pMMO and sMMO have been found to be nonspecific. In the case of MMOs, although CH_4 is the preferred substrate, sMMO will bind and oxidize alkanes up to C-8, as well as ethers, cyclic alkanes, and aromatic hydrocarbons (Colby *et al.*, 1977; Hou *et al.*, 1979; Burrows *et al.*, 1984). pMMO has a narrower substrate range, being able to oxidize alkanes up to C-5, and will not oxidize cyclic alkanes or aromatic compounds (Burrows *et al.*, 1984). The ability of these, and other enzymes to carry out transformations that cells cannot utilize for growth has been termed as cometabolism, i.e., 'the transformation of a nongrowth substrate in the obligate presence of a growth substrate or another transformable compound' (Dalton & Stirling, 1982).

Subsequently, it was discovered that methanotrophic enrichments could degrade priority pollutants such as chlorinated hydrocarbons (Wilson & Wilson, 1985), commonly found in aquifers, landfills, wastewaters, and waste disposal sites across the United States and other countries (Westrick *et al.*, 1984; Semprini, 1997). In the intervening 25 years, a large number of studies have shown that methanotrophs can degrade a large range of halogenated hydrocarbons. Given this ability, methanotrophs have been purposefully stimulated at different sites as: (1) these cells are ubiquitous; (2) the rates of pollutant degradation by these cells is one to two orders of magnitude than that observed for other cells expressing monooxygenases (Oldenhuis *et al.*, 1989); and (3) methanotrophs are easily and selectively stimulated with the provision of a relatively inexpensive and nontoxic substrate (i.e. CH_4) (Semprini & McCarty, 1990; Semprini *et al.*, 1990, 1991; Pfiffner *et al.*, 1997; Iwamoto *et al.*, 2000; Eguchi *et al.*, 2001; Tani *et al.*, 2001; Hazen *et al.*, 2009).

Initial studies focused on methanotrophs expressing sMMO given its broader substrate range, and the belief that pMMO-expressing cells could not degrade chlorinated solvents such as the common degreaser, trichloroethylene, or did so at very slow rates (Oldenhuis *et al.*, 1989; van Hylckama Vleig *et al.*, 1996). An early study by DiSpirito *et al.* (1992), however, showed that pMMO from a variety of methanotrophs could indeed degrade trichloroethylene. Subsequently it was shown that the ability of methanotrophs to degrade compounds such as trichloroethylene substantially increased with increasing Cu availability (Smith *et al.*, 1997; Lontoh & Semrau, 1998). A list of compounds for which the kinetics of degradation by either sMMO or pMMO-expressing methanotrophs is known is shown in Table 5. It should be noted that many other halogenated compounds have been shown to be degraded by methanotrophs, including halogenated aromatics and biphenyls by either purified sMMO or sMMO-expressing cells (Green & Dalton, 1989; Lindner *et al.*, 2000, 2005; Jechorek *et al.*, 2003), as well as some chlorinated ethanes and propanes by pMMO-expressing cells (Oldenhuis *et al.*, 1989), but the kinetics of such degradation have not been reported. It is very likely that as additional studies are performed, this list will be expanded.

Oxidation of compounds such as trichloroethylene is well-known to negatively affect methanotrophic growth due to: (1) competition with CH_4 for binding to MMO; (2) consumption of reducing equivalents; (3) and formation of toxic products, for example, epoxides. Further studies in our laboratory have shown that pMMO-expressing cells in fact

Table 5. Kinetics of halogenated hydrocarbon degradation by methanotrophs known to be expressing either sMMO or pMMO*

	sMMO-expressing cells			pMMO-expressing cells		
Compound	K_s (μM)	V_{max} (nmol min^{-1} mg protein^{-1})	k_1 (mL min^{-1} mg protein^{-1})	K_s (μM)	V_{max} (nmol min^{-1} mg protein^{-1})	k_1 (mL min^{-1} mg protein^{-1})
Methane	92	726	7.9	19	450	25
Halogenated alkanes						
Chloromethane	- - -	- - -	- - -	11	15	1.4
Dichloromethane	4	66	16	73	33	0.5
Dibromomethane	- - -	- - -	- - -	171	45	0.3
Chloroform	34	1100	32	ND	ND	ND
Bromoform	- - -	- - -	- - -	ND	ND	ND
Trifluoromethane	ND	ND	ND	ND	ND	ND
1,2-Dichloroethane	77	130	1.7	- - -	- - -	< 0.06
1,1,1-Trichloroethane	214	48	0.2	ND	ND	ND
1-Chloropropane	- - -	- - -	2.1	- - -	- - -	- - -
1,2-Dichloropropane	- - -	- - -	0.72	- - -	- - -	- - -
1,3-Dichloropropane		- - -	1.16	- - -	- - -	- - -
1,2,3-Trichloropropane	- - -	- - -	0.14	- - -	- - -	- - -
Halogenated alkenes						
Vinyl chloride	160	2100	13	26	42	1.6
trans-Dichlorethylene	148	662	4.5	42	61	1.5
cis-Dichloroethylene	30	364	12.1	0.8	0.12	0.15
1,1-Dichloroethylene	5	12	2.4	2.5	0.23	0.092
Trichloroethylene	145	580	4	7.9	4.1	0.52
Halogenated aromatics						
1,2,3-Trichlorobenzene	- - -	- - -	0.0038	ND	ND	ND
1,2,4-Trichlorobenzene	ND	ND	ND	ND	ND	ND

References: Oldenhuis *et al*. (1991), Sullivan & Chase (1996), van Hylckama Vleig *et al*. (1996), King (1997), Bosma & Janssen (1998), Han *et al*. (1999), Han & Semrau (2000), and Lee *et al*. (2006).

ND, no detectable degradation; - - -, not reported. *Reported units converted where necessary from per gram cells to per mg protein assuming cells are 50% protein.

are able to survive more readily in the presence of mixtures of chlorinated ethenes, and can actually degrade more of these compounds at high concentrations due to the greater specificity of pMMO for CH_4 and relatively slower rates of pollutant transformation to more toxic products (Lee *et al*., 2006; Yoon & Semrau, 2008). As described earlier, it appears that it may be advantageous to emphasize the use of the 'tortoise' or pMMO-expressing methanotrophs over the 'hare' or sMMO-expressing cell as the tortoise, although slow, is able degrade pollutants over a longer time frame than the hare that quickly wanes due to the initial rapid, yet harmful oxidation of these pollutants (Lee *et al*., 2006).

Although methanotrophs have been stimulated for *in situ* bioremediation of polluted sites, the predominant form of MMO expressed by *in situ* communities has been difficult to determine, largely due to the difficulty of quantifying either mRNA levels or specific enzymatic activity in environmental samples (Bælum *et al*., 2008). Phenylacetylene has been found to be a selective inhibitor of sMMO and pMMO activity of a small number of pure methanotrophic cultures (Lontoh *et al*., 2000), and with its use and the finding of structural genes for sMMO in one site at the Idaho National Laboratory, it was concluded that sMMO was expressed and responsible for trichloroethylene degradation at this site (Wymore *et al*., 2007). Although the use of phenylacetylene as a selective inhibitor of sMMO activity is attractive, its efficacy is predicated on having independent assessments of methanotrophic population size as it will also inactivate pMMO-expressing cells at high phenylacetylene : biomass ratios. Additional information also suggests that some pMMO-expressing cells may be sensitive to phenylacetylene (Lee *et al*., 2009).

In a test site at the Department of Energy facility at Savannah River, it was concluded based on increased frequency of detection of sMMO genes and transcripts upon the provision of CH_4 and air, that sMMO was responsible for observed trichloroethylene degradation at this site (Hazen *et al*., 2009). It should be noted that similar assays for the detection of pMMO genes and their transcripts, however, were not mentioned, and apparently not performed. As such, it is difficult to conclude that pMMO-expressing cells were not present and not responsible, at least in part, for the observed trichloroethylene degradation.

Using competitive RT-PCR assays developed in our laboratories (Han & Semrau, 2004), we found that methanotrophic cultures in soil slurries and groundwater from a

mixed chloroethane/chloroethene plume expressed only pMMO, and not sMMO, and concluded that pMMO-expressing cells were responsible for the observed chlorinated hydrocarbon degradation at this site (Forrester *et al.*, 2005).

Greenhouse gas removal

With ongoing concerns as to global warming, a great deal of attention has been paid as how to best reduce anthropogenic emissions of different greenhouse gases, particularly CH_4 from landfills and agricultural soils. Although present in relatively small concentrations in the atmosphere, currently ~1.7 p.p.m.v., CH_4 is approximately 25 times as efficient as carbon dioxide at absorbing infrared radiation (IPCC, 2007) and atmospheric CH_4 concentrations have risen rapidly since the industrial revolution. Landfills in particular are significant sources of CH_4, releasing between 3 and 7×10^{13} g of CH_4 a year (Reay *et al.*, 2007), or 10–20% of the total global annual anthropogenic emission of CH_4 (IPCC, 2007). Despite the significant amounts of CH_4 emitted, it is estimated that anywhere between 10% and 90% of CH_4 produced from methanogenic activity deep within the refuse is actually consumed by methanotrophs before it enters the atmosphere (De Visscher *et al.*, 2007), and occasionally landfills act as CH_4 sinks, i.e., CH_4 is removed from the atmosphere due to high methanotrophic activity in the landfill cover soil (Boeckx *et al.*, 1996; Bogner *et al.*, 1997; Barlaz *et al.*, 2004). In the United States, large landfills are required to actively extract CH_4 that can either be flared or used for energy production (US EPA, 1996), and such strategies can reduce CH_4 emissions to the atmosphere by as much as 90% (Mosher *et al.*, 1999). Despite these systems, landfills in the United States are estimated to release ~4.2×10^{12} g CH_4 a year, or ~24% of all anthropogenic CH_4 emissions in the United States (Energy Information Administration, 2008). Given these phenomena, stimulation of methanotrophic activity in landfill cover soils to reduce fugitive emissions of CH_4 is receiving more attention.

A variety of strategies have been proposed to stimulate methanotrophic activity to reduce CH_4 emissions and are extensively reviewed by others (Huber-Humer *et al.*, 2008; Scheutz *et al.*, 2009). Briefly, engineered systems can be categorized as either 'biocovers' or 'biofilters.' Biocovers involve the application of permeable material, typically organic matter (e.g. compost, sewage sludge, wood chips) over the surface of a landfill. Such materials can have effective gas transport (of both CH_4 from the underlying refuse and oxygen from the atmosphere) while also having adequate water retention to stimulate methanotrophic activity. Laboratory column studies of different biocover materials have been found to allow for removal rates of CH_4 ranging from 22 to 242 g CH_4 m^2 day^{-1} (Scheutz *et al.*, 2009), although lower rates have been reported, particularly at low temperatures and high moisture contents (Kettunen *et al.*, 2006; Einola *et al.*, 2008). Mature organic matter with readily degradable compounds removed to reduce overall oxygen demand was found most appropriate as oxygen consumption by heterotrophs was thereby minimized (Scheutz *et al.*, 2009). Some field trials have been performed that show biocovers can not only reduce CH_4 emissions from landfills, but also result in atmospheric CH_4 uptake (Barlaz *et al.*, 2004). Others have found that biocover performance is reduced in wet seasons, likely due to reduced oxygen transport limiting methanotrophic activity (Jugnia *et al.*, 2008).

Biofilters also attempt to stimulate methanotrophic activity, but are contained fixed bed reactors with biofilms established on some packing material. As with biocovers, appropriate packing materials have sufficient porosity and a high moisture holding capacity to allow for the establishment of methanotrophic growth, although inert materials such as a variety of plastics, silicates, as well as mature organics have been used (Kennes *et al.*, 1996; Alonso *et al.*, 1997; Cox *et al.*, 1997; Okkerse *et al.*, 1999; Du Plessis *et al.*, 2003; Streese & Stegmann, 2003; Wilshusen *et al.*, 2004; Iranpour *et al.* 2005). Biofilters are commonly separate units into which CH_4 gas streams and air are pumped, and in these situations, can also be used for the control of CH_4 emissions from concentrated animal feeding operations (factory farms). As these systems require active pumping systems to introduce both landfill gas and oxygen in landfills, biofilters are occasionally integrated into the landfill cover soil to utilize passive ventilation (Gebert & Gröngröft, 2006).

Laboratory column studies have found CH_4 oxidation rates as high as 28 g CH_4 m^3 h^{-1} when the inlet CH_4 concentration was 300 000 p.p.m.v. (Haubrichs & Widmann, 2006). In many locations, ambient CH_4 concentrations are much lower, for example, immediately above landfills CH_4 concentrations have been reported to range from below detection limits to as high as 8000 p.p.m.v. (Carmen & Vincent, 1998), while in liquid manure storage areas of factory farms concentrations typically range from 140 to 28 000 p.p.m.v. (Melse & Van Der Werf, 2005). Experimental studies indicate that methanotrophic biofilters can remove appreciable amounts at concentrations ranging from 700 to 1500 p.p.m.v. (Melse & Van Der Werf, 2005; Nikiema *et al.*, 2009). Modeling exercises indicate that well-characterized pMMO-expressing cells, i.e., *M. trichosporium* OB3b, can achieve steady-state conditions at a minimum input CH_4 concentration of 500 p.p.m.v., and at this concentration, have removal rates of ~5.7 g CH_4 m^3 h^{-1}. Model results, however, indicate biofilters with *M. trichosporium* OB3b expressing sMMO cannot achieve stable operations until input CH_4 concentrations are at least 3000 p.p.m.v., and then only have removal rates of ~4 g CH_4 m^3 h^{-1} (Yoon *et al.*, 2009a). Collectively these data suggest that engineered

systems can be attractive options for controlling CH_4 emissions, particularly if the cells are expressing pMMO.

It has also been found that methanotrophs also remove significant amounts of CH_4 from the atmosphere, i.e., at concentrations on the order of 1.7 p.p.m.v., orders of magnitude lower than what is found in engineering environments such as landfills and factory farms. Currently it is estimated that such high affinity methanotrophs remove $2.0–6.0 \times 10^{12}$ g CH_4 year^{-1} globally (Henckel *et al.*, 2000a), but it was not until recently that the phylogeny and physiology of these cells was determined, largely due to difficulties in isolating and culturing these cells at the ambient CH_4 concentration (~1.7 p.p.m.v.). Molecular analyses indicated that Type II methanotrophs as determined by the labeling of 18 carbon fatty acids upon provision of < 50 p.p.m.v. ^{14}C-CH_4 were responsible for the observed CH_4 consumption (Holmes *et al.*, 1999; Roslev & Iversen, 1999). *pmoA* gene analyses indicated that these cells likely were novel members of the *Alphaprotoebacteria* (Holmes *et al.*, 1999; Henckel *et al.*, 2000b). Enrichment at 10 000 p.p.m.v. CH_4 led to the isolation of a methanotroph of the *Methylocystaceae* family (Dunfield *et al.*, 1999), with subsequent work at lower CH_4 concentrations (10–100 p.p.m.v.), isolating two *Methylocystis* strains (Knief & Dunfield, 2005). These strains, although unable to grow at atmospheric CH_4 concentrations, were found to oxidize CH_4 at this level for 3 months (Knief & Dunfield, 2005). Interestingly, a third *Methylocystis* strain was found able to grow at 10 p.p.m.v. CH_4, and that this cell had two isozymes of pMMO that had significantly different kinetics and expression patterns (Baani & Liesack, 2008). One isozyme, pMMO1, was only expressed at CH_4 concentrations > 600 p.p.m.v., and had an apparent affinity of CH_4 of 9.3 μM, with an apparent maximal uptake rate of $1.86–2.00 \times 10^{-15}$ mol cell h^{-1}. The other isozyme, pMMO2, was constitutively expressed and had an apparent affinity of CH_4 of 0.11–0.12 μM, with an apparent maximal uptake rate of $0.11–0.13 \times 10^{-15}$ mol cell h^{-1}. More remarkably, pMMO2 isozyme could oxidize CH_4 at atmospheric concentrations. Further studies also indicate that methanotrophs oxidizing CH_4 at atmospheric concentrations in acidic forest soils were expressing pMMO (Kolb *et al.*, 2005). Collectively these data show that pMMO-expressing cells are responsible for atmospheric CH_4 consumption, and it may be possible to utilize their activity for mitigation of global anthropogenic CH_4 emissions.

Production of single-cell protein

The use of microorganisms as an alternative source of protein for both human and animal consumption has been considered since World War I, and interest has increased in recent years as many developing countries struggle to provide diets with sufficient protein to their populations (Kuhad *et al.*, 1997). Microbial protein can be made from yeasts, fungi, algae, and bacteria, including methanotrophs. In the case of the latter, the use of CH_4 for single-cell protein production has the advantage of not using agricultural products as a staring material and continuous-cultivation techniques have been developed that enable the large-scale growth of methanotrophs for protein production. Most notably, industrial efforts by Norferm Danmark A/S in Norway can produce 8000 tons year^{-1} of protein derived from *M. capsulatus* Bath, termed BioProtein, and it is reported that production will be increased to 40 000 tons year^{-1} (Winder, 2004). The use of methanotrophs for production of single-cell protein, however, has the disadvantage that methanotrophic growth can be limited by slow mass transfer of CH_4 from the head space to the liquid space, as well as that CH_4 is sparingly soluble in water. Recent studies, however, have found that the addition of paraffin oil can promote methanotrophic growth by reducing mass transfer limitations (Han *et al.*, 2009). With these and other efforts, the use of methanotrophs for production of single-cell protein is likely to continue.

An additional factor that must be considered, however, is that although the Norferm Danmark A/S bioreactor was designed to stimulate the growth of methanotrophs, subsequent studies found that the system was repeatedly invaded by three different cells, an *Aneurinibacillus* species, a *Brevibacillus agri* strain, and a *Ralstonia* species. It appears that these cells were necessary to establish stable microbial communities as they consumed methanotrophic metabolites that otherwise would have inhibited methanotrophic growth (Bothe *et al.*, 2002). Although in this case the nonmethanotrophic cultures appear to be nontoxic, for example, do not produce enterotoxins, future efforts to utilize methanotrophs, or any other microbial system for single-cell protein production must keep in mind that completely sterile conditions are very difficult to maintain at an industrial scale, and as such close monitoring is required. Although we are not aware of any studies that have examined the use of methanotrophic-derived protein for human consumption, several studies have examined its use as a supplement in the diets of chickens, salmon, minks, and dogs (Müller *et al.*, 2004; Berge *et al.*, 2005; Øverland *et al.*, 2007; Schøyen *et al.*, 2007a, b), and it is likely other uses of methanotrophic-derived protein will be developed in the future.

Factors affecting methanotrophic community structure and activity

Little is known how different environmental conditions affect the distribution, numbers, and activity of methanotrophs other than Type I and II strains. The following

sections highlight the information collected to date, but it should be stressed that similar information should be collected for other groups of methanotrophs to see how these cells respond to varying environmental conditions that result from both seasonal fluctuations and longer-term climate changes.

CH_4

Initial studies found that in agar diffusion columns with counter gradients of CH_4 and O_2, Type II methanotrophs were more prevalent in areas with low O_2 and high CH_4 concentrations while Type I methanotrophs were preferentially found in inverse conditions (Amaral & Knowles, 1995). One study in rice paddies, areas of CH_4 fluxes similar to that in landfills, indicated that Type II methanotrophs also predominated over Type I methanotrophs as CH_4 concentrations increased (Macalady *et al.*, 2002). This study did not definitively show which species were enriched as it focused on signature phospholipid fatty acids and it should be noted that this was in saturated systems that may have an effect on methanotrophic activity and community composition. Another study showed from both PLFA and 16S rRNA gene analyses that both Type I and II methanotrophs were present in significant numbers in rice paddies, but Type II cells again appeared to dominate (Bodelier *et al.*, 2000).

Other studies, however, have found that both types of methanotrophs were active and both contributed to CH_4 oxidation at high CH_4 concentrations (i.e. 10 000 p.p.m.v.), although Type I methanotrophs dominated at 1000 p.p.m.v. CH_4 (Henckel *et al.*, 2000b). Studies using molecular techniques to determine the composition of *in situ* methanotrophic communities in landfills agree with these findings, i.e., using DNA microarrays, both Type I and II methanotrophs were found in landfill cover soils (Bodrossy *et al.*, 2003; Stralis-Pavese *et al.*, 2004; Gebert *et al.*, 2008) where high CH_4 concentrations are common. Finally, using a combination of PCR and denaturing gradient gel electrophoresis, it has been shown that Type I methanotrophs respond more quickly to shifts in CH_4 levels, but that Type II cells had stable populations that became more significant at high CH_4 concentrations (Henckel *et al.*, 2000b). These data suggest that Type I methanotrophs may dominate in heterogeneous environments that support rapid growth, while Type II methanotrophs, being able to withstand such environmental fluctuations, dominate over time, particularly in soils that develop high CH_4 concentrations (Vecherskaya *et al.*, 1993; Henckel *et al.*, 2001).

Moisture content

As discussed above, the possibility that Type II methanotrophs may dominate over time as they may turnover CH_4 more quickly is intriguing, but one must be aware that several other factors have been shown to affect methanotrophic community composition and activity, including moisture content, with the general conclusion that methanotrophic activity has some optimal moisture content. Specifically, high moisture contents have been shown to limit methanotrophic activity, likely due to limited diffusion of CH_4 and air (Whalen & Reeburgh, 1990;Jones & Nedwell, 1993; Bender & Conrad, 1995; Czepial *et al.*, 1996). Not all studies agree, however, as high moisture contents ($> 50\%$) can actually stabilize CH_4 consumption rates, possibly by inducing methanotrophic growth, by lowering dissolved oxygen concentrations, or by making bioavailable other organic compounds that may facilitate methanogenic activity deep in the soil column (Benstead & King, 1997; West & Schmidt, 1998). At low moisture contents where diffusion is not the rate-limiting step on CH_4 availability, methanotrophic activity has also been observed to be inhibited, likely due to increased osmotic stress and/or desiccation (Conrad, 1996; Czepial *et al.*, 1996; Schnell & King, 1996;Jäckel *et al.*, 2001).

From these conflicting findings, it is still not possible to clearly identify the mechanism(s) by which moisture content affects methanotrophic activity. It is possible that the conflicting results reported may be due to different communities with different activity (i.e. different MMO expression) having a competitive advantage under different wetting regimes. Such a hypothesis is supported from the findings of Henckel *et al.* (2001). In this study, drainage of rice field soils revealed that Type I and II methanotrophs were differentially affected by reducing water content. Specifically it was discovered that Type I methanotrophs were more diverse after 8 days of drainage, but not evenly distributed vertically whereas Type II cells were still present throughout soils cores, but their composition was not drastically changed. Such a finding suggests that Type I methanotrophs may be more adaptable to changing environmental conditions, as indicated by other studies where CH_4 and oxygen were varied (Henckel *et al.*, 2000b; Auman *et al.*, 2000).

Temperature

From the limited number of phylogenetic studies done to date on the effect of temperature on methanotrophic communities it appears that Type I methanotrophs dominate at low temperatures in biofilters (Gebert *et al.*, 2003, 2004), and all characterized psychrophilic methanotrophs to date are within the *Gammaproteobacteria*. This conclusion is supported by more recent studies of methanotrophic communities in landfill soils, where it was found that Type I signals were more dominant at 10 °C than 20 °C using PLFA analyses (Börjesson *et al.*, 2004). It has been well-documented, however, that temperature changes typically have little effect on overall methanotrophic activity in soils, with Q_{10}

values typically between 1 and 2 (Whalen *et al.*, 1990; King & Adamsen, 1992; Crill *et al.*, 1994; Roslev *et al.*, 1997; Börjesson *et al.*, 2004). Such low values are attributed to slow mass transfer of CH_4 (Dunfield, 2007), although occasionally higher Q_{10} values are reported in soils that may have higher gas diffusivity (MacDonald *et al.*, 1997; Christophersen *et al.*, 2000) or exposed to CH_4 concentrations $> 10\,000$ p.p.m.v. (De Visscher *et al.*, 2001). At temperature extremes, however, i.e., < 10 and $> 40\,^{\circ}C$, CH_4 oxidation is significantly limited in forest and landfill cover soil samples (Boeckx & Van Cleemput, 1996; Boeckx *et al.*, 1996; Czepial *et al.*, 1996; Whalen & Reeburgh, 1996; Christophersen *et al.*, 2000), likely due to inhibition of mesophilic methanotrophs.

Nitrogen

Another parameter that has been shown to have a range of effects on methanotrophic activity is the availability of nitrogen. Previous research on CH_4 oxidation by *in situ* methanotrophic communities generally agrees that addition of nitrogen as ammonium salts typically reduces the uptake of CH_4 by these cells, either due to competition for binding sites in the methane monooxygenase or through product toxicity (Bédard & Knowles, 1989; Crill *et al.*, 1994; Dunfield & Knowles, 1995; Willison *et al.*, 1995; Tlustos *et al.*, 1998; Nold *et al.*, 1999). Others disagree with this conclusion, suggesting instead that ammonium inhibits methanotrophic activity *in situ* through nonspecific ionic effects (Kightley *et al.*, 1995). This conclusion is supported by the finding that ammonia added as NH_4Cl more significantly inhibited *in situ* CH_4 oxidation in forest soils than did an equimolar amount of ammonia added as $(NH_4)_2SO_4$, possibly due to increased sorption (and thus reduced bioavailability) of ammonium on soils by sulfate (Schnell & King, 1994). Other studies, however, have found that addition of ammonia actually enhances methanotrophic population size and/or activity (Bender & Conrad, 1995; Hilger *et al.*, 2000; De Visscher *et al.*, 2001; Krüger *et al.*, 2001). Combined these findings of both inhibition and enhancement of methanotrophic activity with the addition of ammonia are difficult to explain.

It has been speculated that these contradictory findings may be either due to relief of nitrogen limitation in some situations causing community shifts or some coupling of nitrogen assimilation pathways with CH_4 oxidation causing increased intracellular competition and consumption of reducing equivalents (Bodelier & Laanbroek, 2004). These findings are particularly important for methanotrophic communities and activities in areas such as landfill cover soils as the molar ratio of CH_4 to nitrogen in these locations can be expected to quite high, thus nitrogen may limit the overall methanotrophic community size and composition. In these situations, *in situ* methanotrophic community structure may be dominated by those cells that can fix nitrogen. The range of methanotrophs known to express nitrogenase was initially thought to be limited to Type II strains and *Methylococcus* species, and it is possible that the ability of these strains to fix nitrogen may enable them to predominate in areas with high CH_4/low nitrogen ratios. It is now known, however, that many Type I species can also fix nitrogen (Auman *et al.*, 2001), and thus the relative (in)-ability of members of a methanotrophic community to fix nitrogen can affect overall methanotrophic community size and composition *in situ*.

Subsequent studies have shown that addition of ammonia to rice paddy and forest soils selectively stimulated the growth of Type I methanotrophs (Mohanty *et al.*, 2006). Similarly, the addition of urea to rice paddy soils and ammonium to landfill cover soils has been shown to favor the growth of Type I methanotrophs over Type II methanotrophs (Noll *et al.*, 2008; Lee *et al.*, 2009). Collectively these data suggest that fertilization of environments dominated by Type I will have little effect on CH_4 uptake, but fertilization of environments dominated by Type II methanotrophs could inhibit CH_4 consumption due to changes in the methanotrophic community composition (Mohanty *et al.*, 2006). Attempts to increase CH_4 consumption *in situ* through the addition of nitrogen should consider the initial community composition as well as the ability of individual members to fix nitrogen when considering amending a site with nitrogen.

Copper

Perhaps the most important factor controlling methanotrophic activity is the Cu : biomass ratio. The general effect of Cu on methanotrophic activity, specifically on the relative expression of sMMO and pMMO has been well known for some time (Scott *et al.*, 1981; Dalton *et al.*, 1984; Stanley *et al.*, 1983; Dalton, 2005). Initial studies from H. Dalton's group showed that it was 'possible to manipulate the environmental growth conditions so one form of the enzyme (e.g. MMO) will predominate in the cell' (Stanley *et al.*, 1983). In cells expressing pMMO, Cu has also been shown to control expression up to 55-fold and to alter substrate affinity and specificity (Lontoh & Semrau, 1998; Lontoh, 2000; Choi *et al.*, 2003). Whole-cell CH_4 oxidation by *M. album* BG8 (Type I, can only express pMMO) and *M. trichosporium* OB3b, (Type II, capable of expressing both sMMO and pMMO) were both enhanced with the addition of Cu, but: (1) *M. album* BG8 had higher ($\sim 2\times$) pseudo-first-order rates (V_{max}/K_s) of CH_4 oxidation than *M. trichosporium* OB3b at all Cu concentrations examined, and (2) *M. trichosporium* OB3b expressing pMMO had a higher affinity and pseudo-first-order rates of CH_4 oxidation

rates than when expressing sMMO (Lontoh & Semrau, 1998; Lontoh, 2000). As a result, cells expressing pMMO appear to have a competitive advantage over cells expressing sMMO for turning over CH_4 at low concentrations. Conversely, at high CH_4 concentrations, methanotrophic communities should preferentially express sMMO as the turnover of CH_4 is faster, thus allowing those cells capable of expressing sMMO to grow more rapidly. The importance of Cu appears to be due to the large amount of Cu found in active purifications of pMMO that are believed to be involved in the oxidation and/or electron transport from the *in vivo* reductant to O_2 (Nguyen *et al*., 1994, 1996, 1998; Semrau *et al*., 1995; Zahn & DiSpirito, 1996; Basu *et al*., 2003; Choi *et al*., 2003, 2005; DiSpirito *et al*., 2004; Balasubramanian & Rosenzweig, 2007).

MMO expression *in situ*

pMMO-expressing cells are commonly found in different environments, including peat bogs, landfills and forest, and desert soils (Kolb *et al*., 2005; Chen *et al*., 2007, 2008; Angel & Conrad, 2009). In studies of peat bogs and landfills, no expression of sMMO was found, despite relatively high CH_4 concentrations that would appear to favor sMMO-expressing cells (Chen *et al*., 2007, 2008). At this time, it is not clear what the environmental distribution of sMMO- vs. pMMO-expressing cells might be, or how the interaction of multiple parameters, for example, the ability of methanotrophs to sequester Cu as well as the availability of nitrogen, oxygen, and CH_4 affect *in situ* MMO expression. It is recommended that more attention be paid to this issue to best determine how to manipulate *in situ* methanotrophic activity for enhanced pollutant degradation.

Cu regulation of gene expression and protein synthesis

Seminal work in the Murrell laboratory clearly showed that Cu affected MMO expression at the transcript level, with transcripts of the sMMO gene cluster only expressed at low Cu–biomass ratios (Nielsen *et al*., 1996, 1997; Murrell *et al*., 2000). Subsequently, several genes were found to play key roles in regulating sMMO expression in *M. trichosporium* OB3b, i.e., *rpoN* (encoding for σ^N), *mmoR* (a σ^N-dependent transcriptional activator or enhancer-binding protein), and *mmoG* (encoding a GroEL homologue) (Stafford *et al*., 2003). These genes were also found in *M. capsulatus* Bath, along with *mmoQ* and *mmoS* that have significant identity to two component sensor-regulator systems (Csáki *et al*., 2003) but their precise function is still unknown. Based on these findings, it is proposed that *mmoR* and *mmoG* are expressed in the absence of Cu, and that MmoR then facilitates transcription of the structural genes of sMMO by forming a complex with σ^N. In the presence of Cu, it is postulated that MmoR is inactivated such that effective binding to σ^N does not occur, thereby inhibiting transcription of the *mmo* operon (Trotsenko & Murrell, 2008).

Less is known about the regulatory genes controlling expression of pMMO although the *pmo* operon is known to be transcribed from σ^{70} promoters in a variety of methanotrophs (Gilbert *et al*., 2000; Stolyar *et al*., 2001; Stafford *et al*., 2003). It is hypothesized that *pmo* operon transcription may involve a Cu-binding regulatory protein that derepresses pMMO synthesis by associating with some (as yet unknown) repressor molecule after binding Cu (Murrell *et al*., 2000).

As such, it is still unclear how the sMMO and pMMO gene clusters are reciprocally regulated with respect to Cu. It is known using quantitative RT-PCR assays, *pmoA* is constitutively expressed in *M. capsulatus* Bath, and that the level of expression per cell increases with increasing Cu in the growth medium (Choi *et al*., 2003). It is possible that the recently characterized Cu-specific-binding compound in methanotrophs, methanobactin, may be the mechanism by which Cu is sensed by these cells and may be involved in coordinating the reciprocal regulation of sMMO and pMMO (see below for more information on methanobactin).

In addition to the expression of the two MMOs, Cu also has been found to regulate the proteome of *M. capsulatus* Bath, including: (1) the expression of at least two of the four formaldehyde dehydrogenases (Stirling & Dalton, 1978; Vorholt *et al*., 1998; Zahn *et al*., 2001; Vorholt, 2002); (2) the development of internal membranes (Stanley *et al*., 1983; Dalton *et al*., 1984; Prior & Dalton, 1985a, b; Collins *et al*., 1991; Peltola *et al*., 1993; Brantner *et al*., 1997; Choi *et al*., 2003); (3) hemerythrin (Karlsen *et al*., 2005; Kao *et al*., 2008); and (4) several outer membrane proteins that appear to be involved in Cu assimilation, regulation or transport (Berson & Lidstrom, 1997; Fjellbirkeland *et al*., 1997, 2001; Karlsen *et al*., 2003; Helland *et al*., 2008). However the mode by which Cu regulates overall gene expression in methanotrophs remains vague.

Cu-specific uptake systems in methanotrophs

As methanotrophs respond strongly to varying Cu availability and cells expressing pMMO have a high demand for Cu, these cells must have an effective mechanism to sense and collect Cu. Such a mechanism must be able to compete with the other Cu complexing agents present in surface and subsurface soil systems, especially as many methanotrophs depend on pMMO for CH_4 oxidation. Specifically, in subsurface environments, Cu bioavailability can be effectively decreased via association with organic matter (e.g.

humic acids), and sorption to the surfaces of metal oxide soils (Morton *et al.*, 2000b).

The first indication of a specific Cu uptake system was provided from phenotypic characterization of the constitutive sMMO mutants (sMMO^C) in *M. trichosporium* OB3b isolated by Phelps *et al.* (1992). Phenotypic characterization of the sMMO^C mutants demonstrated the mutants were defective in Cu uptake and also showed evidence for an extracellular Cu-complexing agent (Fitch *et al.*, 1993).

Subsequent studies have shown that the extracellular Cu-complexing agent is the Cu-binding compound (i.e. methanobactin) first identified in association with pMMO of *M. capsulatus* Bath (Zahn & DiSpirito, 1996). Furthermore, it has been discovered that methanobactin accumulates in the growth medium of *M. trichosporium* OB3b grown in the presence of $< 0.7\,\mu M$ Cu (DiSpirito *et al.*, 1998). Cu-containing methanobactin (Cu-mb) is rapidly internalized into the cell when Cu was provided at concentrations between 0.7 and 1.0 μM (Fig. 4), which coincides with repression of sMMO expression and induction of higher levels of pMMO expression (Zahn & DiSpirito, 1996; Choi *et al.*, 2003, 2005). These results all suggest that in *M. trichosporium* OB3b, methanobactin is the extracellular component of a Cu uptake system, and that methanobactin is analogous to iron siderophores in other bacteria. These results also suggest methanobactin is involved in regulating expression of the two MMOs by those cells that can express both forms.

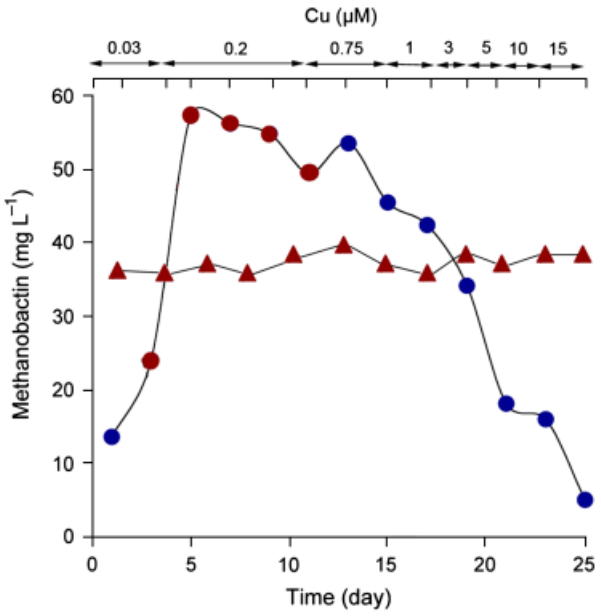

Fig. 4. Effect of Cu concentration in the media on the extracellular concentration of methanobactoin in wild type *Methylosinus trichosporium* OB3b (•) and sMMO^C mutants *M. trichosporium* OB3b PP319 (▲) in sequential batch reactors. Cells were initially cultured in no amended media (i.e. media containing 0.03 μM Cu), then the Cu concentration sequentially increased to a final concentration of 15 μM Cu by replacing 80% of the medium every two days. Symbols in red represent cultures with cells expressing sMMO and those in blue represent cultures with cells exclusively expressing pMMO. Scale on top represents the initial Cu concentration following replacement of growth medium.

Physiological characterization of the sMMO^C mutants isolated by Phelps *et al.* (1992) also provided additional evidence for a methanobactin-based Cu acquisition system (DiSpirito *et al.*, 1998). In contrast to wild-type *M. trichosporium* OB3b, as shown in Fig. 4, these sMMO^C mutants constitutively showed a high concentration of methanobactin in the culture medium regardless of the Cu concentration in the culture medium. Also in contrast to wild-type *M. trichosporium* OB3b, Cu-mb could be isolated from the extracellular fraction, but not from the whole cell or washed membrane fractions (DiSpirito *et al.*, 1998). The results suggest the sMMO^C mutants were defective in some component of the Cu-mb uptake machinery. With the exception of the lack of pMMO expression, the sMMO^C mutants showed no negative effects on growth or respiration as Cu concentrations were varied. These mutants acquired 15–20% of the Cu taken up by wild-type cells and showed normal respiration and growth rates suggesting a separate Cu acquisition system (DiSpirito *et al.*, 1998).

A second Cu-regulated Cu uptake system has been detected in *M. capsulatus* Bath (Berson & Lidstrom, 1996, 1997; Fjellbirkeland *et al.*, 1997, 2001; Karlsen *et al.*, 2003; Helland *et al.*, 2008). This Cu acquisition system is based on the extracellular Cu-binding protein, MopE or CorA, which is negatively regulated by Cu. MopE is a structurally novel protein which coordinates Cu via two histidines, a kynurenine (an oxidation product of tryptophan) and an axial H_2O (Helland *et al.*, 2008). These results suggest that the MopE/CorA Cu uptake system may be a 'house-keeping' Cu acquisition system and the methanobactin-based system is a more specialized Cu acquisition related to pMMO synthesis.

Methanobactin – a Cu-specific-binding compound

Many recent advances have been made on the structural, spectral and physiological properties of methanobactin produced by *M. trichosporium* OB3b. Biochemical and mass spectroscopic analyses studies indicate Cu-containing methanobactin is a small chromopeptide that contains one Cu^+ per molecule via a novel S & N coordination (Kim *et al.*, 2004; Behling *et al.*, 2008; Fig. 5). Methanobactin can bind either Cu^+ without a change in the oxidation state or Cu^{2+} which is subsequently reduced to Cu^+ (Choi *et al.*, 2006). In the absence of Cu, methanobactin will bind a variety of metals, including Fe, Ni, Zn, Au, Ag, Pb, Mn, Cd, and Co (Choi *et al.*, 2008). However, with the exception of Ag, Au, and Hg, subsequent exposure to Cu results in metal

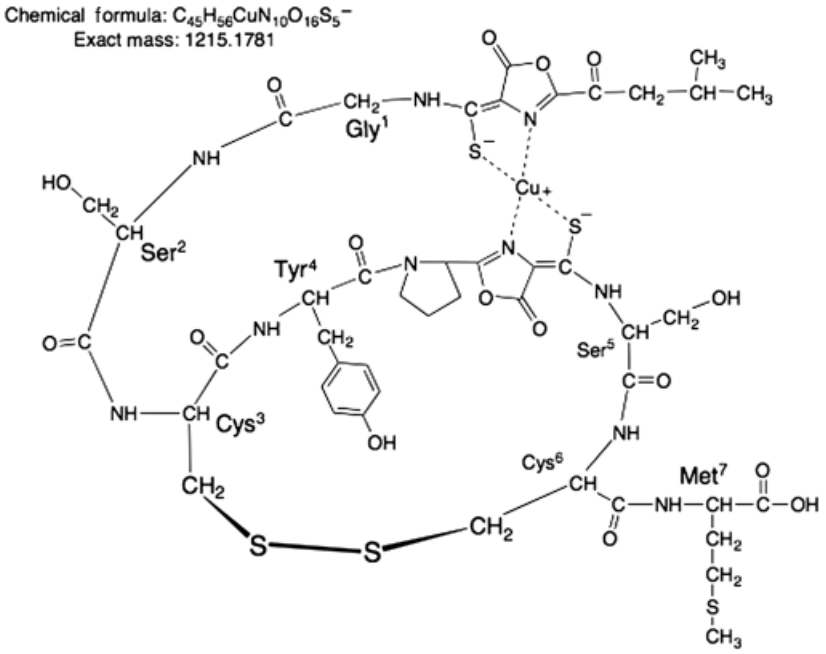

Fig. 5. Proposed structure for methanobactin from *Methylosinus trichosporium* OB3b (Courtesy of Warren Gallagher).

displacement by Cu. The methanobactin from *M. trichosporium* OB3b has also been shown to reduce two atoms of either Cu^{2+} or Au^{3+} to Cu^{+} and Au^{0}, respectively, although only one metal atom remains associated with methanobactin (Choi *et al.*, 2006, 2008).

Using isothermal calorimetry analyses, it was discovered that methanobactin from *M. trichosporium* OB3b binds Cu as a tetramer with an initial binding constant of $3.3 \times 10^{34} \pm 3.0 \times 10^{11}\,M^{-1}$, ~17–19 orders of magnitude greater than that of other metals bound by methanobactin (Choi *et al.*, 2006). Competition studies against known Cu-binding compounds with binding constants as high as 10^{30} were used to confirm this very high Cu-binding constant (Smith & Martell, 1975, 1989; Martell & Smith, 1984; Choi *et al.*, 2006). At Cu(II) to methanobactin ratios above 0.25 the Cu is coordinated as a dimer and the binding constant dropped to $2.6 \pm 0.46 \times 10^{8}$, followed by coordination as a monomer with a binding constant of $1.4 \pm 0.2 \times 10^{6}$ at Cu to methanobactin ratios above 0.5 Cu per methanobactin. All other metals had secondary binding constants one to four orders of magnitude lower. The initial high binding constant followed by lower binding constants at higher Cu to methanobactin ratios is consistent with its potential role in the acquisition of Cu from the environment. Given these data, it is apparent that methanobactin from *M. trichosporium* OB3b is the first example of a chalkophore ('chalko' is Greek for copper; Kim *et al.*, 2004), or extracellular microbial mechanism to specifically collect Cu from the environment.

Subsequent to the purification of methanobactin in *M. trichosporium* OB3b, it was isolated from the spent growth medium of *M. capsulatus* Bath and *M. album* BG8 (Choi *et al.*, 2008), implying that the ability to produce chalkophores is wide-spread in methanotrophs. By modifying the chrome azurol S assay used for detecting siderophore production (Schwyn & Neilands, 1987), we have developed a plate assay to screen chalkophore production by methanotrophs that can be extended to determine the ability of other groups of cells to express a chalkophore (Yoon *et al.*, 2009b). Using this plate assay, we confirmed that *M. album* BG8 and *M. capsulatus* Bath do secrete a chalkophore, but that *Methylocystis parvus* OBBP does not. It is interesting to note that chalkophore production is seen in both *Gammaproteobacteria* methanotrophs (*M. album* BG8 and *M. capsulatus* Bath) and *Alphaproteobacteria* methanotrophs (*M. trichosporium* OB3b), and that the ability to express a chalkophore is not dependent on whether the cell can express sMMO or not (*M. album* BG8 and *M. parvus* OBBP do not have the genes for sMMO, while *M. trichosporium* OB3b and *M. capsulatus* Bath do). These findings suggest that methanotrophic distribution along redox gradients may reflect a cell's (in)ability to express a high affinity Cu uptake system, i.e., a chalkophore. It is possible that in environments where Cu bioavailability is limited (e.g. low redox conditions where Cu(I) sulfides are likely to form) those cells that are able to express chalkophores have a competitive advantage over these cells that do not. Conversely, in high redox environments where Cu(II) complexes are more readily available, greater methanotrophic diversity may be seen, with cells having lower affinity Cu uptake systems becoming more competitive.

At this time, it is unknown if other methanotrophs, particularly the acidiphilic *Methylocella* and *Methylocapsa* strains as well as the *Verrucomicrobia* methanotrophs make methanobactin. It may be that in the acidic environments from which these strains were isolated, high affinity Cu uptake systems are not necessary as Cu may be readily available given that Cu solubility increases with decreasing pH.

Collectively, these results suggest a Cu uptake system in methanotrophs that is mediated by a molecule or molecules analogous to iron siderophores in other bacteria, but unique in the ability to meet the demand for large quantities of Cu. There are several different pathways known to be involved in synthesis of secondary metabolites, including: (1) ribosomal peptide (RP) synthesis; (2) nonribosomal peptide (NRP) synthesis; (3) nonribosomal-independent siderophore (NIS) biosynthesis pathway; and (4) polyketide biosynthesis pathway (Staunton & Weissman, 2001; Challis, 2005; Walsh & Nolan, 2008; McIntosh *et al.*, 2009). More rarely, hybrids of these pathways are used as well, as is the case for bleomycin synthesis (Shen *et al.*, 2001, 2002; Pfeifer *et al.*, 2003). Because methanobactin is a secondary metabolite with a modified peptide backbone, ribosomal and NRP synthesis pathways are more likely, as NIS and polyketide biosynthesis do not involve peptide linkage between monomeric units.

Behling *et al.* (2008) proposed a reaction pathway for modification of *M. trichosporium* OB3b methanobactin from its hypothetical precursor with Leu–Ser–Gly–Ser–Cys–Tyr–Pro-Ser–Ser–Cys–Met sequence and it may be that methanobactin is a RP either chromosomally or plasmid encoded. In most cases, RPs are expressed as precursor peptides with leader peptides, which are then removed via proteolysis (McIntosh *et al.*, 2009). This proteolysis reaction is mediated by various proteases including serine proteases, cysteine proteases attached to ABC transporters, and metal proteases. Another generally observed feature in RPs is the heterocyclic motif, usually in form of oxazoline/oxazole or thiazoline/thiazole, which is the post-translational modification of Cys, Ser, or Thr. In general, enzymes involved in heterocyclization include a zinc-binding protein, a probable docking protein with ATPase/GTPase activity, and an oxidase (Milne *et al.*, 1999; Zamble *et al.*, 2000). Disulfide bonds such as that seen in methanobactin are also often observed in RPs (Paik *et al.*, 1998; Fontaine & Hols, 2008). In mature RPs, Cys–Cys disulfide bonds have the primary function of rigidifying the structure into the active state. Protein disulfide isomerases (PDI) are often engaged in RP syntheses, and genes similar to known PDI genes have been found in the genome of *M. capsulatus* Bath, for example, MCA0575, MCA2602, and MCA1041 (Ward *et al.*, 2004). It is unknown, however, what if any role these genes play in methanobactin synthesis.

NRP synthesis is the most common pathway for synthesis of siderophores with peptide backbone structures (Crosa & Walsh, 2002; Schwarzer *et al.*, 2003). Instead of being directly translated from mRNA, these peptides are assembled by nonribosomal peptide synthases (NRPS). These enzymes are organized in modules, which are units for addition of each amino acid to an expanding peptide chain. Each module is made up of domains with different functions, which work together for proper addition of a specific amino acid to peptide chains. The basic scheme for elongation of peptide chains in NRPS involves adenylation (A) domains, peptidyl carrier protein (PCP) domains, and condensation (C) domains usually arranged in A–PCP–C manner (Mootz *et al.*, 2002; Schwarzer *et al.*, 2003). A gene has been discovered in *M. capsulatus* Bath that putatively encodes a NRPS (Ward *et al.*, 2004) The putative NRPS has adenylation, thiolation, and acetyltransferase domains, typical of extracellular metal-binding agents (Crosa & Walsh, 2002). Other genes have been found nearby with domains for condensation as well as a terminal thioesterase that combined with the NRPS, may create a charged peptide that can bind a variety of metals, including Cu.

Metal centers of pMMO

Laboratories studying the pMMO agree that the pMMO is a Cu-containing enzyme, composed of three polypeptides with approximate molecular masses of 45 000 Da (α-subunit, *pmo*B), 26 000 Da (β-subunit, *pmo*A), and 23 000 Da (γ-subunit, *pmo*C) (Zahn & DiSpirito, 1996; Takeguchi *et al.*, 1998; Basu *et al.*, 2003; Chan *et al.*, 2004, 2007; DiSpirito *et al.*, 2004; Choi *et al.*, 2005; Dalton, 2005). Both the crystal and electron microscope structures of pMMO from *M. capsulatus* Bath and *M. trichosporium* OB3b showed that pMMO is a trimer $(\alpha\beta\gamma)_3$ (Kitmotto *et al.*, 2005; Balasubramanian & Rosenzweig, 2007) In the crystal structures from *M. capsulatus* Bath and *M. trichosporium* OB3b, each αβγ monomer was modeled to contain either a dinuclear or mononuclear Cu center located at the membrane–periplasm interface of the α-subunit (PmoB) as well as a metal-binding site located within the membrane and coordinated by both the β-subunit (PmoA) and γ-subunits (PmoC) as shown in Fig. 6. The second conserved metal-binding site identified in both structures was occupied by Cu in pMMO from *M. trichosporium* OB3b and zinc in pMMO from *M. capsulatus* Bath. The finding of different metals in this metal-binding site suggests this site may be a labile metal binding site. A mononuclear Cu center was also detected in the periplasmic region of α-subunit (PmoB) in the structure from *M. capsulatus* Bath. However, the mononuclear Cu site was absent in the *M. trichosporium* OB3b structure (Hakemian *et al.*, 2008). The possible

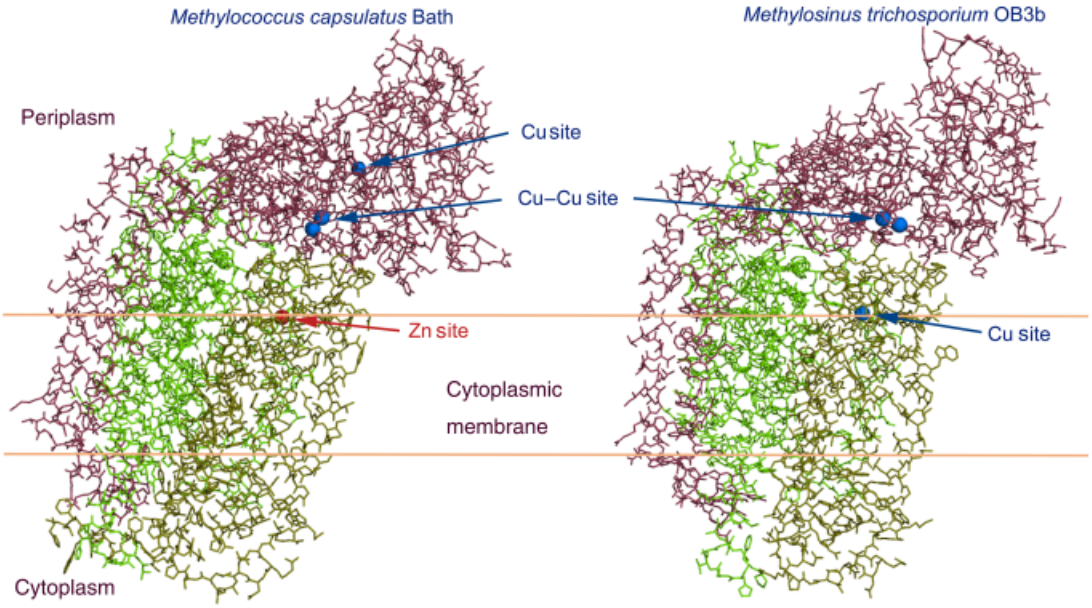

Fig. 6. Crystal structure and membrane orientation of the αβγ monomer of pMMO from *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b. Membrane orientation was predicted using the methodology of Lomize *et al*. (2006) using structural information from Lieberman & Rosenzweig (2005) and Hakemian *et al*. (2008). Subunit colors: α, raspberry; β, chartreuse; γ, deep olive.

function of each observed metal center is described in more detail below.

Mononuclear Cu center

Of the three metal centers identified in the pMMO from *M. capsulatus* Bath, the mononuclear Cu-binding site is the least likely to be involved in CH_4 oxidation. The site was absent in the *M. trichosporium* OB3b crystal structure and sequence information demonstrates the site is not well conserved, and thus an unlikely site for CH_4 oxidation (Hakemian *et al*., 2008; Rosenzweig, 2008; Semrau *et al*., 2008).

Dinuclear Cu center

This metal center was identified in both *M. trichosporium* OB3b and *M. capsulatus* Bath within the α-subunit (PmoB) and has been modeled as a dinuclear Cu center. The Cu–Cu distance was short, however, approximately 2.6 Å, and both Cu atoms are coordinated by only three His (Rosenzweig, 2008). In addition, considering the resolution of the crystal structure (2.8 Å), this site could be modeled as a mononuclear Cu center, a possibility put forward by Lieberman & Rosenzweig (2005). In fact, the spectral properties of this site are more in keeping with a mononuclear Cu site (Yuan *et al*., 1997, 1998, 1999). As a dinuclear Cu center, this center has been proposed as a potential active site (Lieberman & Rosenzweig, 2005; Hakemian *et al*., 2008; Rosenzweig, 2008). However, this Cu center in pMMO differs from other dinuclear copper oxidases in which the two Cu atoms are coordinated by six His and show greater Cu–Cu distances (Yuan *et al*., 1998, 1999). It is noteworthy that, although the three His involved in Cu coordination are conserved in the PmoB sequences in methanotrophs from the α- and β- subdivisions of the *Proteobacteria*, the coordinating ligands are absent in the sequences of the three PmoB genes from one of the recently isolated *Verrucomicrobia* methanotrophs originally termed *A. fumarolicum*, now proposed as a strain of *Methylacidiphilum infernorum* (Pol *et al*., 2007; Semrau *et al*., 2008; Op den Camp *et al*., 2009). In this methanotroph, His 137 and His 139 are replaced with Pro and Gly, respectively, and His 33 is absent.

The third or variable metal-binding site

Both Zn and Cu have been detected in the metal-binding site coordinated by amino acids from PmoA and PmoC, suggesting a metal labile site (Lieberman & Rosenzweig, 2005; Hakemian *et al*., 2008). The enzyme preparations used in the crystal structures showed little to no activity, which can result with metal loss and/or replacement at this site. The lack of activity in these preparations also opens the possibility this labile metal-binding site may be occupied by a different metal *in vivo*. Our recent Mössbauer studies provide strong evidence for the presence of a diiron center in active preparations of pMMO and this metal-binding site contains the predicted amino acids necessary to coordinate a diiron center (Martinho *et al*., 2007). As shown in Fig. 7, the

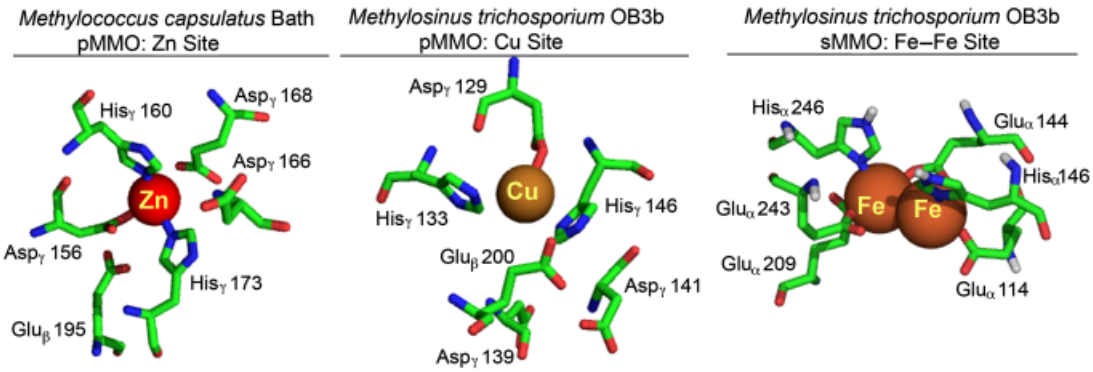

Fig. 7. (a) Potential coordination of a diiron center in the Zn-binding site of pMMO from *Methylococcus capsulatus* Bath (Lieberman & Rosenzweig (2005), the Cu-binding site from *Methylosinus trichosporium* OB3b (Hakemian *et al.*, 2008), and the coordination ligands for the diiron center in the sMMO from *M. trichosporium* OB3b (c) (Elango *et al.*, 1997).

site contains several conserved amino acids (a combination of histidines and amino acids with carboxylate residues) associated with PmoA and PmoC that are similar to that found in the active site of sMMO (Fox *et al.*, 1993; Rosenzweig *et al.*, 1993; Elango *et al.*, 1997). These amino acids are conserved in *all* known pMMO sequences including the pMMO sequences from *Verrucomicrobia* methanotrophs (Semrau *et al.*, 2008). The results from Mössbauer studies of pMMO from *M. capsulatus* Bath suggested to us that active pMMO contains a diiron center (called doublet 1) similar to that observed for the sMMO (Martinho *et al.*, 2007). Most interestingly, the amount of doublet 1 iron in our samples correlates with the activity observed in purified pMMO and in pMMO from washed membrane and whole cell samples as shown in Fig. 8.

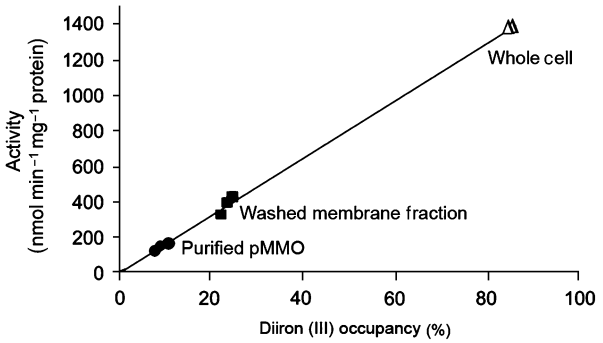

Fig. 8. Activity of different pMMO preparations vs. diiron site occupancy. Activity was measured by the oxidation of propylene to propylene oxide (rates from Martinho *et al.*, 2007 and D.W. Choi *et al.*, unpublished data).

Models of pMMO structure

Although there is general agreement that pMMO is a Cu-containing trimer, as discussed above, there is wide-spread disagreement on the number, type, and function of metal centers associated with the pMMO as well as the nature of the physiological electron donor. All the models agree that the Cu requirement is high, i.e. approximately 10-fold higher than the Cu requirement observed in other microorganisms (Nguyen *et al.*, 1994, 1996, 1998; Zahn & DiSpirito, 1996; Choi *et al.*, 2003), but four different models for the pMMO have been proposed. These models are described in more detail below and are outlined in Table 6.

The first model is based on the crystal structures of the enzyme described above which found either two or three metal-binding sites in pMMO from *M. trichosporium* OB3b (Hakemian *et al.*, 2008) or *M. capsulatus* Bath (Lieberman & Rosenzweig, 2005), respectively. In this model the putative dinuclear Cu center was suggested as the site of CH_4 oxidation, although the possibility that the site occupied by either zinc or Cu in the crystal structures could be the locus of CH_4 oxidation was not ruled out. The reader is referred to several recent reviews for more details on Model I (Balasubramanian & Rosenzweig, 2007; Hakemian & Rosenzweig, 2007; Rosenzweig, 2008). Additional evidence for Model I comes from the recent studies on small-molecule ligand–Cu complexes (Himes & Karlin, 2009a, b; Woertink *et al.*, 2009). These studies demonstrated that ligand–Cu complexes are catalytically capable of the selective oxidation of CH_4 to methanol without further oxidation of methanol.

Model II or the trinuclear Cu cluster model is based on the work from Sunny Chan's group (Nguyen *et al.*, 1994, 1996, 1998; Chan *et al.*, 2004, 2007). The model proposes the pMMO contains 15 Cu atoms per αβγ subunit of which 12 Cu atoms are arranged into either trinuclear catalytic (C-clusters) or trinuclear electron transfer (E-clusters) clusters, plus a mononuclear and a dinuclear Cu site identified in the crystal structure of pMMO from *M. capsulatus* Bath. The locations of the C-clusters have not been identified.

Table 6. Characteristics of different models of pMMO content and reductant source

	Model			
Property	I	II	III	IV
Metals				
Cu $\alpha\beta\gamma^{-1}$	3	15	2–3	2–3
Fe $\alpha\beta\gamma^{-1}$	0.75	0.17	1–2	1.2
Cu-mb $\alpha\beta\gamma^{-1}$	0	0	0	6–8
Activity*	17	21.5	$\approx 40^{\dagger}$	160
Physiological rate (%)	1.1	1.4	2.7	10.7
Reductant	Ubiquinol	NADH/ ubiquinol	MDH ferrocytochrome	Ubiquinol/ Cu-mb
Active site	Dinuclear Cu cluster	Trinuclear Cu center	Unidentified	Diiron center

*Highest activity reported by in purified pMMO preparations upon which the respective models are based, rates in nmol propylene oxidized $\cdot min^{-1}\, mg^{-1}$ protein.

†Partial purification, purified with methanol dehydrogenase.

Using N- and C-termini fragments of the α-subunit of pMMO from *M. capsulatus* Bath expressed in *Escherichia coli*, Chan's group proposed the E-clusters are associated with the soluble C-terminal region of PmoB (Yu *et al.*, 2003). This model proposes that the E-clusters accept electrons directly from NADH and transfer them to the C-clusters where CH_4 oxidation is presumed to occur. The reader is referred to the reviews by Chan *et al.* (2004, 2007) for a detailed description of Model II.

Model III, proposed by Dalton and colleagues, suggests that pMMO contains two to three Cu and one to two Fe atoms per αβγ subunit (Basu *et al.*, 2003; Kitmotto *et al.*, 2005; Myronova *et al.*, 2006). This group also proposed that the $(\alpha\beta\gamma)_3$ trimer making up pMMO can be considered a hydroxylase (pMMO-H) that is assembled with the methanol dehydrogenase (MDH) in a super complex (pMMO-C) in which the MDH serves as the reductase for pMMO-H possibly via a *c*-type cytochrome (Kitmotto *et al.*, 2005).

Like Model III, Model IV proposes that the pMMO contains two Cu and two Fe atoms per αβγ subunit, which is also considered to be a hydroxylase component of a super complex (Zahn & DiSpirito, 1996; Martinho *et al.*, 2007). The two models differ in the nature of the reductases in that Model IV proposes the pMMO-H is coupled to the respiratory chain at the cytochrome bc_1 complex possibly via ubiquinol. Model IV also proposes that pMMO-H is coordinated with Cu-mb which may serve to shuttle electrons to the pMMO. Lastly, and perhaps most significantly, in Model IV the pMMO contains a diiron center very similar to that observed in sMMO (DiSpirito *et al.*, 2004; Choi *et al.*, 2005, 2008; Martinho *et al.*, 2007). This diiron center is believed to be located where zinc and Cu site coordinated by amino acids from PmoA and PmoC in *M. capsulatus* Bath and *M. trichosporium* OB3b, respectively.

Supporting evidence for a diiron-containing pMMO

Based on metal composition, pMMO models can be grouped into either 'Cu-only enzyme' (Models I and II) or a 'Cu and iron enzyme' (Models III and IV). As described, above, many reviews and theoretical manuscripts supporting a Cu-only enzyme have been published (Chan *et al.*, 2004, 2007; Shimokawa *et al.*, 2006; Yoshizawa & Shiota, 2006; Hakemian & Rosenzweig, 2007; Hakemian *et al.*, 2008; Rosenzweig, 2008; Himes & Karlin, 2009a, b). However, a summary of the evidence for a Cu and iron enzyme has not been provided, and is presented here.

Like most researchers in the field we initially held the belief that the pMMO was a Cu-only enzyme (Shiemke *et al.*, 1991; Semrau *et al.*, 1995). However, in the last 15 years, results from several laboratories are more consistent with the Fe–Cu models (Zahn & DiSpirito, 1996; Takeguchi *et al.*, 1998; Choi *et al.*, 2003, 2005; Dalton, 2005; Tumanova *et al.*, 2008). The major observations pointing to an iron–Cu enzyme include the following. First, the higher activity preparations from different laboratories contained one to two Fe iron atoms associated with the αβγ subunit of pMMO (Table 2). Second, the Mössbauer parameters of doublet 1 in purified preparations of pMMO are identical to those observed in the sMMO (Martinho *et al.*, 2007). Third, the concentration of doublet 1 in purified pMMO preparations, washed membrane samples and in whole cells from *M. capsulatus* Bath expressing the pMMO correlates with activity (see Fig. 8). In fact, assuming the percentage of iron in doublet 1 in studies from proponents of Models I and II is similar to preparations shown in Fig. 8, the concentrations of iron in these samples are sufficient to support the reported activities. The observation of a labile diiron center is similar to early sMMO studies that also showed a correlation between iron content and activity (Colby & Dalton, 1978; Woodland & Dalton, 1984; Fox *et al.*, 1988, 1989). Fourth, the variable metal binding site or 'Zn-binding' site of the pMMO and the environment of the diiron center in the sMMO are very similar (Fig. 7). Fifth, the amino acids suspected to coordinate the putative diiron center are conserved in all known pMMO sequences (Semrau *et al.*, 2008). This is in contrast to the mono- and dicopper-binding sites of pMMO, which are absent in the predicted amino acid sequence of *pmoB* from acidophilic methanotrophs (Pol *et al.*, 2007). Sixth, an $S = 9/2$ $Fe^{III}Fe^{IV}$ intermediate in pMMO-H samples is detected when these are incubated with hydrogen peroxide (Zahn & DiSpirito, 1996; Tumanova *et al.*, 2008). Seventh, the active sites of all characterized enzymes that oxidize saturated aliphatic

compounds contain either a mono- or dinuclear iron center (Tumanova *et al.*, 2008).

It has been suggested that iron and resulting Mössbauer spectra in pMMO preparations result from contaminating heme, iron–sulfur proteins or hemerythrin (Nguyen *et al.*, 1998; Chan *et al.*, 2004, 2007; Karlsen *et al.*, 2005; Hakemian & Rosenzweig, 2007; Kao *et al.*, 2008; Rosenzweig, 2008). In contrast, several studies have shown that the increased iron content in active pMMO preparations was not associated with heme or other UV-visible absorption or electron paramagnetic resonance (EPR) quantifiable iron-containing proteins and instead was associated with the pMMO fractions (Zahn & DiSpirito, 1996; Choi *et al.*, 2003; Martinho *et al.*, 2007). In particular, UV-visible absorption spectra of purified pMMO preparations from our laboratory have demonstrated repeatedly that heme concentrations were < 0.005 hemes per $\alpha\beta\gamma$ protomer, eliminating heme as a source of the diiron signal. $[4Fe–4S]^{2+}$ can be excluded via quantification by EPR analysis and by observing that pMMO-H has only one cysteine residue, Cys 92 on the α subunit (Martinho *et al.*, 2007). The soluble diiron-containing hemerythrin with a molecular mass of 14 800 Da has been shown to be a Cu-regulated protein (Karlsen *et al.*, 2005). However, Cu induction of hemerythrin is low in comparison with the $\alpha\beta\gamma$ polypeptides of pMMO-H, and we have never observed this soluble polypeptide in washed membrane fractions or in purified pMMO preparations as determined using spectral analysis and denaturing protein gels (Fig. 9). Thus, even if present at trace levels, the concentrations of cytochromes or hemerythrin were well below detection concentrations via Mössbauer spectroscopy and can also be excluded as the source of the diiron signal.

Further support for Models III and IV comes from acetylene-binding studies of pMMO and sMMO. Acetylene is a suicide substrate of both MMOs (Prior & Dalton, 1985c; Lontoh *et al.*, 2000). Both enzymes are believed to oxidize acetylene to a reactive intermediate such as an epoxide that binds irreversibly to the enzyme. In the sMMO, acetylene has been shown to bind to the diiron-containing 54 000 Da polypeptide (Prior & Dalton, 1985c). In pMMO, acetylene binds almost exclusively to PmoA (one of the polypeptides involved in coordination of the putative diiron cluster) and shows little if any association with PmoB (the polypeptide containing the dicopper-binding site) (Prior & Dalton, 1985b; DiSpirito *et al.*, 1992; Zahn & DiSpirito, 1996).

Taken together the results suggest the site of CH_4 oxidation occurs at the diiron site (Model IV) coordinated by amino acids from PmoA and PmoC. This site is the only metal coordination site conserved in all known pMMO sequences and the similarity to the CH_4 hydroxylation site in the sMMO makes it a predictable site for CH_4 oxidation in pMMO. Unfortunately, definitive resolution of this and other differences between the models will require improvements to existing pMMO preparations. All of the studies on purified pMMO samples have been carried out on samples representing 11% or less of whole-cell physiological activity, with Cu-only models based on samples showing $< 1\%$ of the physiological rate. Thus, although much of the data are informative, the results are often conflicting and should be interpreted with the quality of the enzyme preparation in mind.

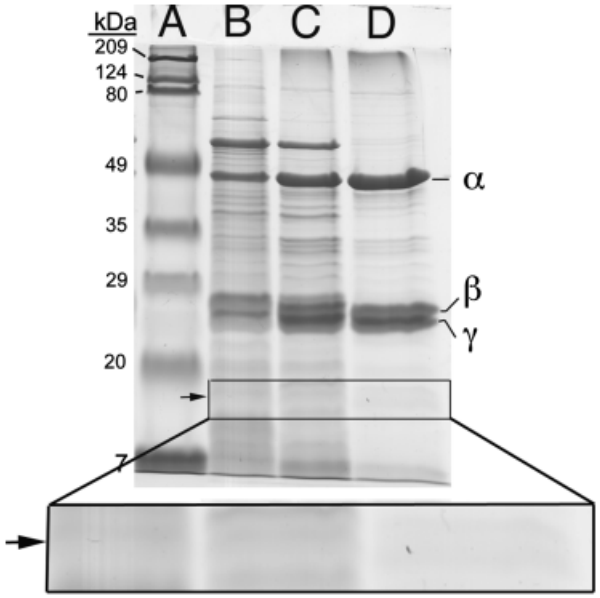

Fig. 9. SDS-denaturing gel of molecular mass standards (A), whole cell sample of *Methylococcus capsulatus* Bath cultured in NMS media supplemented with 80 μM Cu and 40 μM Fe^{57} (B), washed membrane fraction of cells from lane B (C), and isolated pMMO (D). Left, enlargement of the 7000–20 000-Da region illustrating the absence of detectable concentrations of haemerythrin, arrow at 14 800 Da (molecular mass of hemerythrin).

Role of methanobactin in CH_4 oxidation by pMMO

In addition to the number and type of metal centers associated with the pMMO, researchers working in this field disagree on the initial electron donor for the enzyme (Table 6). A number of studies have suggested that the pMMO is coupled to the respiratory chain at the quinone or bc_1-complex (Stirling *et al.*, 1979; DiSpirito *et al.*, 2004 and references therein). However, both NADH and duroquinol (a quinone analog) have been used as electron donors in purified preparations of pMMO-H (Shiemke *et al.*, 1995; Nguyen *et al.*, 1998; Takeguchi *et al.*, 1998; Cook & Shiemke, 2002; Basu *et al.*, 2003; Choi *et al.*, 2003; Chan *et al.*, 2007). Results from the Dalton group have also suggested that the enzyme may be coupled to the MDH possibly via a soluble *c*-type cytochrome (Myronova *et al.*, 2006) as recently

observed in the ammonia monooxygenase (Gilch *et al.*, 2009). If the results from the different laboratories are correct, it would follow that *in vitro* pMMO samples can accept electrons from a variety of reductants. The presence of Cu-mb in pMMO preparations could explain these apparent contradictory results. Studies on the redox and catalytic properties of Cu-mb show that Cu-mb is reduced by a variety of reductants including NADH, duroquinol, and a number of *c*-type ferricytochromes (Choi *et al.*, 2008; A.A. DiSpirito, unpublished data). It is possible that Cu-mb acts as a shuttle to direct electrons from the *in vivo* reductant to the dinuclear Cu site, with subsequent transfer to the diiron site for CH_4 oxidation. The redox potential for methanobactin has not been determined, but it is estimated that the redox potential for methanobactin from *M. trichosporium* OB3b is 0 to +100 mV. Thus, based on this redox potential, Cu-mb could serve as a redox mediator between the quinol or cytochrome bc_1 complex and pMMO-H. It should be stressed, however, that this estimate is based only on which metals are reduced by methanobactin from *M. trichosporium* OB3b and which metals are bound without a change in redox state (Choi *et al.*, 2006), and should be more accurately determined.

Future research directions

In this review, we have provided a summary of the known phylogenetic and physiological diversity of methanotrophs, with a major focus on (1) applications of methanotrophs for pollutant degradation greenhouse gas removal and protein production (2) the role of Cu in regulating methanotrophic activity, (3) the mechanism(s) of Cu uptake identified in methanotrophs, and (4) the metal centers within pMMO. Although much has been learned in the past 10–15 years that has greatly expanded our understanding of methanotrophic diversity and physiology, there are still unresolved issues that merit more attention.

Methanotrophs are found in a wide range of environments. However, the presence of methanotrophs in extreme environments and the finding of *Verrucomicrobia* methanotrophs indicate that more efforts should be extended to isolate and characterize methanotrophs from more diverse environments, particularly targeting methanotrophs with high affinity MMOs. With this information, it may be possible to utilize methanotrophs for various environmental applications, for example, the use of the (thermo)acidophilic methanotrophs for enhanced biodegradation at mixed waste sites and the stimulation of high affinity methanotrophs for greater atmospheric CH_4 removal. Given the importance of CH_4 as a greenhouse gas, future work should examine how changing land use affects overall *in situ* methanotrophic activity, particularly how such land use affects the magnitude of this CH_4 sink.

On a related topic, more extensive environmental surveys of methanotrophic gene expression should be performed to better determine when and where sMMO is the predominant form of MMO expressed by methanotrophic communities. As discussed here, only a limited number of studies have examined what form of MMO is expressed *in situ*, and in the many of these studies, only pMMO expression was evident. It is unclear under what *in situ* conditions one would expect sMMO expression, or how, if one wished, could alter different geochemical parameters (other than perhaps Cu bioavailability) to induce expression of either sMMO or pMMO.

The discovery of methanobactin in at least three methanotrophs suggests that this is a widespread, but not universal mechanism used by methanotrophs for Cu sequestration. We recommend that methanobactin production be characterized using a broader range of methanotrophs. Such information can help address several important questions. For example, does the ability to produce, secrete, and take up methanobactin enable those cells to survive in lower redox conditions where Cu bioavailability is more likely to be limiting due to formation of insoluble Cu complexes? Does the ability to synthesize and secrete methanobactin thus help determine methanotrophic community structure? Do acidophilic methanotrophs produce methanobactin? These cells appear to be very sensitive to trace metals, and it may be that such active Cu uptake mechanisms may not be necessary, given the greater solubility (and by extension, availability) of Cu in acidic conditions. Furthermore, as members of the acidophilic genus *Methylocella* do not express pMMO, Cu uptake may not be critical for these cells' metabolism. By extension, do closely related cells such as ammonia-oxidizing bacteria produce methanobactin? It has been shown that *Nitrosomonas europaea*, although unable to produce siderophores, takes up siderophores produced by other cells (Wei *et al.*, 2006). As the addition of Cu has been shown to enhance the activity of the ammonia monooxygenase in cell-free extracts, but not in whole cells (Ensign *et al.*, 1993), do ammonia-oxidizing bacteria, and possibly other cells, rely on methanobactin production by methanotrophs for Cu acquisition?

Similarly, much information is lacking as to the genetics of methanobactin production. Efforts should be supported to determine the mechanism by which methanobactin is synthesized. By doing so, this could also help determine any role methanobactin may have in coordinating sMMO/pMMO expression in those cells that can express both forms of MMO, as well as role of methanobactin in pMMO.

Finally, as noted by Ob den Camp, although the terms 'Type I' and 'Type II' were initially meant to denote groupings based on both physiology and phylogeny, but are now commonly used to indicate either *Gamma-* or *Alphaproteobacteria* methanotrophs, such a distinction may now be

more confusing than helpful. This is particularly true given not only the discovery of *Verrucomicrobia* methanotrophs, but also that not all known *Proteobacteria* methanotrophs fit neatly into these categories. First, *Methylocella* and *Methylocapsa*, although grouping in *Alphaproteobacteria*, are clearly different from other methanotrophic genera in this class. Second, several methanotrophs in both the *Gamma-* and *Alphaproteobacteria* have signature fatty acids of both Type I and Type II methanotrophs, for example, *Methylocystis heyeri* (*Alphaproteobacteria*), *M. crimeensis* (*Gammaproteobacteria*), and *M. thermalis* (*Gammaproteobacteria*) (Heyer *et al*., 2005; Tsubota *et al*., 2005; Dedysh *et al*., 2007). Given the vast phylogenetic and physiological diversity of methanotrophs found in the past 10 years, and the possibility that with high throughput metagenomic and culturing techniques applied to different ecosystems more methanotrophic diversity will be discovered, it may be appropriate to expand the current nomenclature to consider more types (e.g. Type III, IV, etc.) to have greater distinction between different methanotrophic groups. Alternatively, it may be better to discard such classifications completely in favor of more consistent and meaningful phylogenetic descriptions as suggested by Op den Camp *et al*. (2009).

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