Methanotrophy – Environmental, Industrial and Medical Applications

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Abstract

Aerobic methanotrophs are an intriguing group of microbes with the singular ability to consume methane as their sole source of carbon and energy. As such, methanotrophs are receiving increased attention to control methane emissions to limit future climate change. Methanotrophs have a wide range of other applications, including pollutant remediation and methane valorization (e.g. conversion of methane to protein, bioplastics, and biodiesel amongst other products). Methanotrophs also produce a novel copper-binding compound – methanobactin – that has significant potential for the treatment of copper-related human pathologies. Here we provide an overview of aerobic methanotrophy, describe current and future applications of these unique microbes, as well as discuss various strategies one can consider to better realize the opportunities these microbes present.

Introduction

Aerobic methanotrophs, or methane-oxidizing bacteria, are a group of microbes with great environmental and industrial importance. For example, methanotrophs are well known to play a key role in controlling the net emission of methane from soils, a potent greenhouse gas with a global warming potential ≈34 times that of carbon dioxide over a 100-year time frame (Myhre *et al.*, 2013). In fact, it is estimated that as much as 90% of methane generated in anaerobic soils via methanogenesis may be removed via methanotrophy (Chowdhury and Dick, 2013). Further, methanotrophs oxidize methane under ambient temperatures and pressures, and thus are attractive platforms for the valorization of methane to products such as single-cell protein, bioplastics, and biofuels (Semrau *et al.*, 2010; Khmelenina *et al.*, 2015; Strong *et al.*, 2015, 2016). Interest in commercial application of methanotrophy has dramatically accelerated in recent years as methane prices have become quite low, with the industrial price of natural gas dropping from \$13.06 per 1000 ft^3 in July 2008 to \$3.92 per 1000 ft^3 in November 2017 (United States Energy Information Administration, 2018). Herein we provide an overview of aerobic methanotrophy followed by a discussion of current and developing applications.

Overview of methanotrophic diversity

Aerobic methanotrophs are distinguished from other microorganisms by their ability to utilize methane as their sole carbon and energy source, yet are phylogenetically and physiologically quite diverse. Although most characterized aerobic methanotrophs can be considered mesophilic, i.e. pH and temperature optima around 7 and 30°C, respectively, there are many examples of aerobic methanotrophs living under more 'extreme' conditions. That is, acidophilic (growth at pH < 3), alkaliphilic (growth at pH > 9) thermophilic (growth above 50°C), psychrophilic (growth below 15°C) as well as halophilic (growth at salt

concentrations >1 M) methanotrophs have been isolated (Op den Camp *et al.*, 2009; Semrau *et al.*, 2010; Knief, 2015).

Perhaps not surprisingly then, aerobic methanotrophs exhibit very broad phylogenetic diversity, with extant strains grouping with the Proteobacteria, Verrucomicrobia, and NC10 phyla. More precisely, 20 genera have been characterized in the γ-*Proteobacteria* class, six genera in the α-*Proteobacteria* class, two genera in the Verrucomicrobia phylum and one in the NC10 phylum (Table 1.1). It is quite likely that as more researchers focus on methanotrophy, the phylogenetic and physiological diversity of these intriguing microbes will greatly expand. In this review, however, we will focus on the *Proteobacteria* methanotrophs as these microbes are ubiquitous, play critical roles in mitigating greenhouse gas emissions *in situ*, and we have a greater depth of knowledge of the genetics, biochemistry and metabolism of these strains. We hasten to stress, however, that undoubtedly other methanotrophs possess intriguing properties with great environmental and industrial relevance.

A key enzyme in aerobic methanotrophy – methane monooxygenase

As noted above, methanotrophs are noted by their ability to utilize methane as their sole source of carbon and energy. Doing so, however, requires a remarkable enzyme that converts methane to methanol, the methane monooxygenase. Cleaving the C–H bond in methane is inherently challenging given the high bond dissociation energy of the C–H bond in methane – 104 kcal/mol. What is perhaps even more remarkable is that aerobic methanotrophs have not one, but two forms of methane monooxygenase. One form of the enzyme,

Table 1.1 General phylogeny of aerobic methanotrophs[a]

Phylogeny	Reference
Phylum	
Gammaproteobacteria	
Family	
Methylococcaceae	
Genera	
Clonothrix[b]	Vigliotta *et al*. (2007)
Methylobacter	Whittenbury *et al*. (1970)
Methylocaldum	Bodrossy *et al*. (1997)
Methylococcus	Foster and Davis (1966)
Methylogaea	Geymonat *et al*. (2011)
Methyloglobulus	Deutzmann *et al*. (2014)
Methylomagnum	Khalifa *et al*. (2015)
Methylomarinum	Hirayama *et al*. (2013)
Methylomicrobium	Bowman *et al*. (1995)
Methylomonas	Brown *et al*. (1964)
Methyloparacoccus	Hoefman *et al*. (2014)
Methyloprofundus	Tavormina *et al*. (2015)
Methylosarcina	Wise *et al*. (2001)
Methylosoma	Rahalkar *et al*. (2007)
Methylosphaera	Bowman *et al*. (1997)
Methylovulum	Iguchi *et al*. (2011a)

Table 1.1 Continued

Phylogeny	Reference
Family	
Methylothermaceae	
Genera	
Methylohalobius	Heyer *et al*. (2005)
Methylomarinovum	Hirayama *et al*. (2014)
Methylothermus	Bodrossy *et al*. (1999)
Family	
Crenotrichaceae	
Genera	
Crenothrix[b]	Stoecker *et al*. (2006)
Phylum	
Alphaproteobacteria	
Family	
Methylocystaceae	
Genera	
Methylocystis	Whittenbury *et al*. (1970)
Methylosinus	Whittenbury *et al*. (1970)
Family	
Beijerinkiaceae	
Genera	
Methylocella	Dedysh *et al*. (2000)
Methylocapsa	Dedysh *et al*. (2002)
Methyloferula	Vorobev *et al*. (2011)
Methyloaffinis[b]	Pratscher *et al*. (2018)
Phylum	
Verrucomicrobia	
Family	
Methylacidiphilaceae	
Genera	
Methylacidiphilum	Op den Camp *et al*. (2009)
Family	
Unclassified[c]	
Genera	
Methylacidimicrobium	van Teeseling *et al*. (2014)
Phylum	
NC10	
Family	
Unclassified	
Genera	
Methylomirabilis[b]	Ettwig *et al*. (2010)

[a]For the sake of brevity, methanotrophic species are not listed. For a thorough list/description of validated aerobic methanotrophic species, the reader is directed to (Knief, 2015); [b]No type strains have been isolated/purified, thus these genera should be considered Candidatus; [c]Using a cut-off of 86.5% to distinguish bacteria of different families (Yarza *et al*., 2014), *Methylacidimicrobium* can be considered to be a member of the *Methylacidiphilaceae* family as its 16 rRNA sequence is 89–90% identical to that of *Methylacidiphilum* (van Teeseling *et al*., 2014).

the particulate methane monooxygenase (pMMO) is found in most known methanotrophs (Table 1.2) and is located in the cytoplasmic membrane (Stanley *et al.*, 1983; Dalton *et al.*, 1984; Zahn and Dispirito, 1996; Murrell *et al.*, 2000; Basu *et al.*, 2003; Choi *et al.*, 2003; Lieberman *et al.*, 2003;

Table 1.2 Occurrence of genes encoding for subunits of pMMO, sMMO, or pXMO as well methanobactin biosynthesis in the genomes of select methanotrophs. pMMO – *pmoA;* sMMO – *mmoX*; pXMO – *pxmA*; methanobactin – *mbnB/C*

Strain	Class	*pmoA*	*mmoX*	*pxmA*	*mbnB/C*
Methylobacter tundripaludum SV96	Gammaproteobacteria	Yes	No	Yes	No
Methylobacter marinus A45	Gammaproteobacteria	Yes	No	Yes	No
Methylobacter tundripaludum 21/22	Gammaproteobacteria	Yes	No	Yes	No
Methylobacter luteus IMV-B-3098	Gammaproteobacteria	Yes	No	Yes	No
Methylobacter tundripaludum 31/32	Gammaproteobacteria	Yes	No	Yes	No
Methylobacter whittenburyi ACM	Gammaproteobacteria	Yes	No	Yes	No
Methylobacter sp. BBA5.1	Gammaproteobacteria	Yes	No	Yes	No
Methylocaldum szegediense O-12	Gammaproteobacteria	Yes	No	No	No
Methylococcus capsulatus str. Bath	Gammaproteobacteria	Yes	Yes	No	No
Methyloglobulus morosus KoM1	Gammaproteobacteria	Yes	No	Yes	No
Methylohalobius crimeensis 10Ki	Gammaproteobacteria	Yes	No	No	No
Methylomarinum vadi strain IT-4	Gammaproteobacteria	Yes	No	No	No
Methylomicrobium alcaliphilum	Gammaproteobacteria	Yes	No	No	No
Methylomicrobium album BG8	Gammaproteobacteria	Yes	No	Yes	No
Methylomicrobium buryatense 5G	Gammaproteobacteria	Yes	Yes	No	No
Methylomicrobium agile ATCC 35068	Gammaproteobacteria	Yes	No	Yes	No
Methylomonas methanica MC09	Gammaproteobacteria	Yes	Yes	No	No
Methylomonas sp. MK1	Gammaproteobacteria	Yes	Yes	Yes	No
Methylomonas sp. 11b	Gammaproteobacteria	Yes	Yes	Yes	No
Methylomonas sp. LW13	Gammaproteobacteria	Yes	Yes	Yes	No
Methylomonas denitrificans FJG1	Gammaproteobacteria	Yes	No	Yes	No
Methylosarcina fibrata AML-C10	Gammaproteobacteria	Yes	No	No	No
Methylosarcina lacus LW14	Gammaproteobacteria	Yes	No	No	No
Methylovulum miyakonense HT12	Gammaproteobacteria	Yes	Yes	No	No
Methylocapsa acidiphila B2	Alphaproteobacteria	Yes	No	No	No
Methylocapsa aurea KYG	Alphaproteobacteria	Yes	No	No	No
Methylocella silvestris BL2	Alphaproteobacteria	No	Yes	No	No
Methylocystis sp. SB2	Alphaproteobacteria	Yes	No	Yes	Yes
Methylocystis sp. SC2	Alphaproteobacteria	Yes	No	No	Yes
Methylocystis rosea SV97	Alphaproteobacteria	Yes	No	Yes	Yes
Methylocystis sp. ATCC 49242 Rockwell	Alphaproteobacteria	Yes	No	No	No
Methylocystis parvus strain OBBP	Alphaproteobacteria	Yes	No	No	Yes
Methyloferula stellata AR4	Alphaproteobacteria	No	Yes	No	No
Methylosinus sp. LW4	Alphaproteobacteria	Yes	Yes	No	Yes
Methylosinus trichosporium OB3b	Alphaproteobacteria	Yes	Yes	No	Yes
Methylosinus sp. LW3	Alphaproteobacteria	Yes	Yes	No	Yes
Methylosinus sp. PW1	Alphaproteobacteria	Yes	Yes	No	Yes
Methylocystis sp. LW5	Alphaproteobacteria	Yes	Yes	No	Yes

Lieberman and Rosenzweig, 2005). Another form, the soluble methane monooxygenase (sMMO), is found in some methanotrophs (Table 1.2) and is located in the cytoplasm (Stirling *et al.*, 1979; Stanley *et al.*, 1983; Dalton *et al.*, 1984; Fox *et al.*, 1989; Stainthorpe *et al.*, 1989, 1990a,b; Lipscomb, 1994; Wallar and Lipscomb, 1996). The sMMO has relatively high turnover but poor affinity for methane. sMMO also has a broad substrate range and as a result has significant potential in biocatalysis (Colby *et al.*, 1977; Tonge *et al.*, 1977; Trotsenko and Murrell, 2008; Semrau *et al.*, 2010; Semrau, 2011; Kalyuzhnaya *et al.*, 2015). pMMO, conversely, has relatively low turnover but greater affinity for methane, and so has greater significance for the removal of atmospheric methane (Lee *et al.*, 2006). Interestingly, methanotrophic growth via pMMO is energetically more efficient than growth via sMMO (Trotsenko and Murrell, 2008; Kalyuzhnaya *et al.*, 2015). The operons for both sMMO (*mmo*) and pMMO (*pmo*) are shown in Fig. 1.1, with most genes known to encode for polypeptides of either MMO. The only exceptions are *mmoR* and *mmoG*, known to encode for a regulatory element of the *mmo* operon and a GroEL homologue, respectively (Stafford *et al.*, 2003), and *mmoD*, that plays a critical role in controlling the relative expression of the two forms of MMO for those methanotrophs that can express both sMMO and pMMO (Semrau *et al.*, 2013; Yan *et al.*, 2016).

In addition to the canonical forms of MMO, there is evidence for a third form, or pXMO (Tavormina *et al.*, 2011). This third MMO appears to be a divergent form of pMMO, and has a different gene organization, i.e. *pxmABC* versus *pmoCAB* (Fig. 1.1). Many (but not all) γ-Proteobacteria methanotrophs have been found to harbour these genes, but to date only a small number of α-Proteobacteria methanotrophs appear to have the *pxm* operon, e.g. *Methylocystis rosea* SV97 and *Methylocystis* strain SB2 (Table 1.2 (Ghashghavi *et al.*, 2017; Gu, 2017)). It is unclear what this significance of pXMO may be. Although expression is evident (Tavormina *et al.*, 2011; Kits *et al.*, 2015), it is typically quite low and its role is still undecided in methanotrophic metabolism. It may be, as speculated by Tavormina *et al.* (2011), that pXMO promotes methanotrophic fitness by extending the range of substrates these methanotrophs can utilize for growth and/or serving to remove various toxins. Alternatively, it has been suggested that expression of *pxm* genes increase under hypoxia and in the presence of nitrate. As a result, pXMO may play a role in helping methanotrophs deal with oxygen limitation (Kits *et al.*, 2015). There is still much to be learned, however, as what environmental conditions induce expression of *pxm* genes and if these are in any way coordinated with expression of other MMO genes.

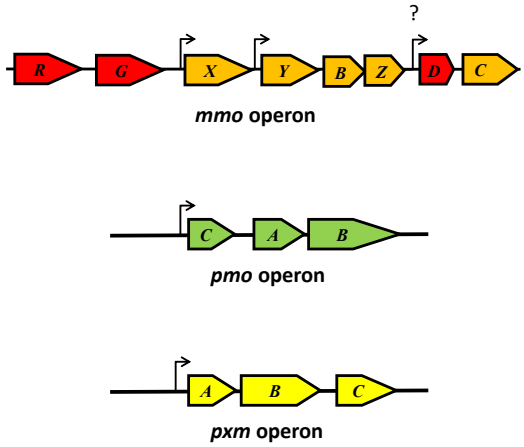

Figure 1.1 *mmo*, *pmo*, and *pxm* operons. Known regulatory genes shown in red.

Methanotrophic response to copper

Aerobic methanotrophs are sensitive to copper, and it is a key factor regulating expression of genes encoding for polypeptides of sMMO and pMMO (Stanley *et al.*, 1983; Dalton *et al.*, 1984; Prior and Dalton, 1985a,b; Choi *et al.*, 2003; Han and Semrau, 2004; Semrau *et al.*, 2010, 2013). *mmoX* is expressed only under low-copper conditions while *pmoA* expression increases substantially when copper is increased. Further, no activity is found in the soluble (cytoplasmic) fraction above 1 μM copper (i.e. no sMMO activity), and as the copper concentration increases, MMO activity in the membranes and whole-cells increases substantially (i.e. increased pMMO activity, Fig. 1.2). Such findings can be explained as sMMO is known to have a diiron complex in its active site, while copper occupies at least two of three or one of two metal centres found in purified pMMO (Lipscomb, 1994; Lieberman *et al.*, 2003; Balasubramanian *et al.*, 2010; Semrau *et al.*, 2010). Interestingly, addition of copper can also affect cell composition.

That is, when *Methylococcus capsulatus* Bath is grown in the absence of copper (and expressing sMMO), a substantial fraction of cell carbon is found as polyhydroxybutyrate (Choi *et al.*, 2003). As copper is added, however, the distribution of cell carbon changes with polyhydroxybutyrate content decreasing and intracytoplasmic membrane (lipid) content increasing. As such, one may be able to use copper to control cell composition and increase either the production of a bioplastic precursor (polyhydroxybutyrate) or a biodiesel precursor (lipid) as described later in this chapter.

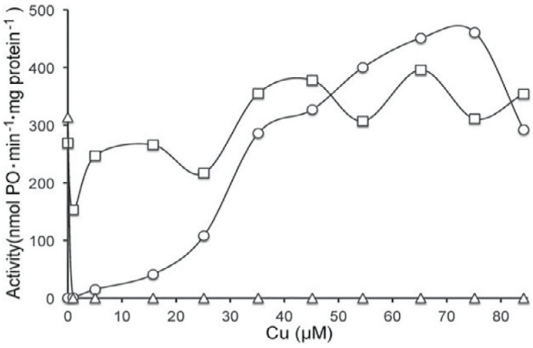

Figure 1.2 MMO activity in *M. capsulatus* Bath as a function of copper. (□): whole cells, (△): soluble fraction, (○): membrane fraction (DiSpirito, unpublished data). PO = propylene oxide.

Copper uptake in methanotrophs

Clearly methanotrophs sense and respond to copper in their environment, but it was only recently that the mechanism(s) used by methanotrophs to do so was discovered. Some aerobic methanotrophs utilize an intriguing copper-binding compound analogous to a siderophore, dubbed a chalkophore ('chalko' is Greek for copper). The first structurally characterized chalkophore, methanobactin, was from the α-*Proteobacteria*, *Methylosinus trichosporium* OB3b (Kim *et al.*, 2004) and is a modified polypeptide with two oxazolone rings with thioamide groups that are responsible for copper binding (Fig. 1.3). Subsequently methanobactin has been isolated and purified from a variety of methanotrophs and is found to have several general characteristics, including being a small (less than 1300 Da) modified polypeptide chain with two heterocyclic rings, either an imidazole, oxazolone or pyrazinedione ring, each with an associated thioamide group that together are responsible for copper binding (Kim *et al.*, 2004; Choi *et al.*, 2006b; Behling *et al.*, 2008; Krentz *et al.*, 2010; El Ghazouani *et al.*, 2011, 2012; Bandow *et al.*, 2012; Vorobev *et al.*, 2013; DiSpirito *et al.*, 2016; Kenney *et al.*, 2016). Methanobactin

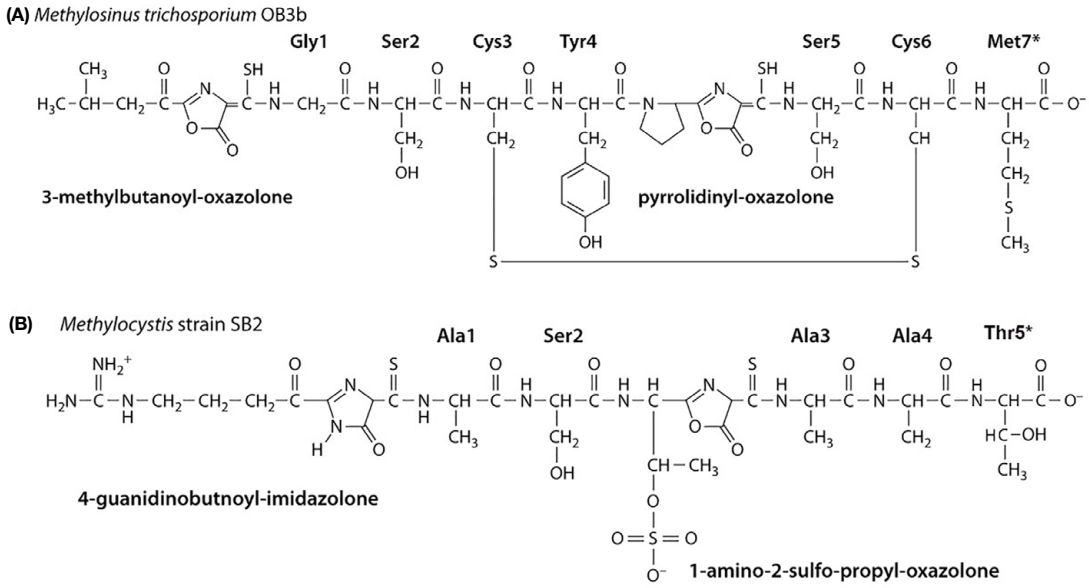

Figure 1.3 Representative primary structures of group I (A) and group II (B) methanobactins. Amino acids marked with * are occasionally absent.

has an extremely high copper-binding constant (Kim *et al.*, 2004; Behling *et al.*, 2008; Krentz *et al.*, 2010; El Ghazouani *et al.*, 2011, 2012; Bandow *et al.*, 2012; Vorobev *et al.*, 2013). Methanobactins can be divided into two general groups on the basis of the types of heterocyclic rings present as well as the presence/absence of a sulfate group (Fig. 1.3). Specifically, Group I methanobactins have two oxazolone rings while Group II methanobactins have a C-terminal oxazolone ring, with the other ring being either imidazolone or a pyrazinedione. Group I methanobactins also contain Cys residues in the mature peptide, while those in Group II do not. The N-terminal amino acid in Group I methanobactin is deaminated, while N-terminal deamination is not observed in Group II methanobactins (Gu *et al.*, 2017). Finally, Group II methanobactins have an associated sulfate group that has not been found to date in Group I methanobactins (Krentz *et al.*, 2010; El Ghazouani *et al.*, 2012; DiSpirito *et al.*, 2016).

Biochemical analyses indicated that methanobactin could be formed from a polypeptide precursor with the heterocyclic rings derived from various amino acids, primarily cysteines (Krentz *et al.*, 2010). Interrogation of available methanotrophic genomes found one possible candidate gene, *mbnA*. Deletion of *mbnA* in *M. trichosporium* OB3b showed that it indeed encodes for the precursor of methanobactin and that it is part of an operon (Fig. 1.4; Semrau *et al.*, 2013), with many genes of unknown function (involved in ring formation), an extrusion protein (that may serve to secrete methanobactin), as well as an aminotransferase recently shown to de-aminate the N-terminal leucine of methanobactin from *M. trichosporium* OB3b, required for subsequent formation and/or stability of the 3-methylbutanoyl-oxazolone moiety (Gu *et al.*, 2017). Downstream of *mbnN* (encoding for the aminotransferase) is another gene cluster that encodes for a di-haem cytochrome peroxidase and its partner protein that may be involved in methanobactin synthesis (Semrau *et al.*, 2018). Upstream of *mbnA* is another gene cluster under the control of a separate promoter with genes encoding for a TonB-dependent transporter (*mbnT*) responsible for methanobactin uptake (Gu *et al.*, 2016), as well as a putative membrane sensor (*mbnR*) and an extracytoplasmic function sigma factor (*mbnI*). A similar system is frequently found in siderophore synthesis where an outer membrane transporter binds a ferri-siderophore, transmitting a signal to a membrane sensor that then activates an extracytoplasmic function sigma factor. This ultimately induces expression of genes required for siderophore synthesis, as well as in some cases genes unrelated to siderophore production or uptake, e.g. genes encoding for exotoxins and proteases (Crosa, 1997; Lamont *et al.*, 2002; Visca *et al.*, 2002; Mahren and Braun, 2003; Braun *et al.*, 2006; Grosse *et al.*, 2007; Brooks and Buchanan, 2008). MbnI may thus be involved in regulating expression of the methanobactin gene cluster and/or of other gene clusters, e.g. the *mmo* and *pmo* operons and perhaps others as yet unknown. Interestingly, interrogation of available microbial genomes indicated that both methanotrophs and non-methanotrophs have similar methanobactin gene clusters as that found in *M. trichosporium* OB3b, suggesting that methanobactin-like compounds may be widespread in nature (Haft *et al.*, 2013; Kenney and Rosenzweig, 2013; Semrau *et al.*, 2013). To date, however, *mbn* genes have been only found in methanotrophs grouping in the α-Proteobacteria, suggesting that γ-Proteobacteria methanotrophs (that also require copper) utilize a different system for copper

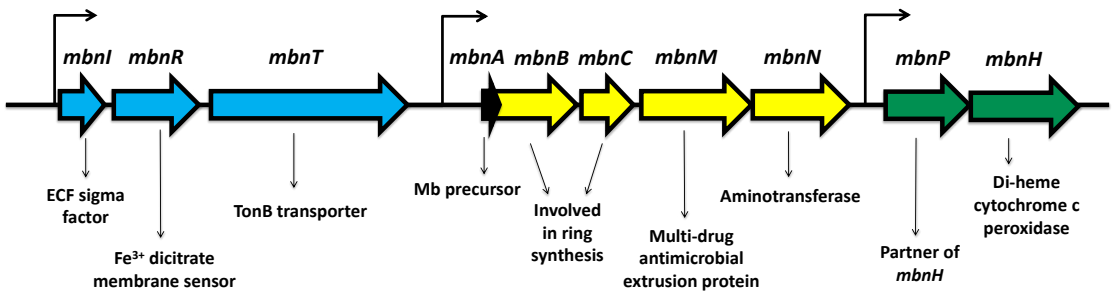

Figure 1.4 Methanobactin gene cluster from *M. trichosporium* OB3b.

sequestration. For example, the γ-Proteobacteria methanotroph, *M. capsulatus* Bath, appears to rely on a surface-bound protein with a tryptophan converted to a kynurenine (MopE) and a secreted form of it (MopE*) for copper binding (Fjellbirkeland *et al.*, 1997; Karlsen *et al.*, 2003; Helland *et al.*, 2008; Ve *et al.*, 2012), although there is some evidence of a methanobactin-like compound in this methanotroph that has not been extensively characterized (Zahn and Dispirito, 1996; Choi *et al.*, 2005). Interestingly, many γ-Proteobacteria methanotrophs have TonB-dependent transporters similar to MbnT, and it may be that some γ-Proteobacteria methanotrophs act as 'cheaters' where they utilize methanobactin made by other methanotrophs to satisfy copper requirements.

Environmental applications/ implications of methanotrophy

Organic pollutant degradation

Given the ubiquity of methanotrophy in terrestrial, freshwater and marine environments, there has been interest for decades to harness the activity of these microbes to address a number of environmental issues, initially for pollutant degradation. The utility of methanotrophs for bioremediation, particularly for organic pollutants is enhanced as both pMMO and sMMO are relatively non-specific and will oxidize a number of compounds in addition to methane. Early work showed that sMMO can degrade a wide range of organic compounds, i.e. alkanes up to C_8, as well as ethers, cyclic alkanes, and aromatic hydrocarbons (Colby *et al.*, 1977; Burrows *et al.*, 1984). pMMO can oxidize alkanes up to C_5, but not aromatic compounds (Burrows *et al.*, 1984). Further, both forms of MMO can attack ethenes, especially chlorinated ethenes that are commonly found at hazardous waste sites (Zahniser, 2015). Alkanes are general converted to the corresponding alcohol (with the hydroxyl group primarily at the terminal carbon), while aromatic hydroxylation occurs at the meta position (Strong *et al.*, 2017). Alkenes are oxygenated across the carbon-carbon double bond, with the subsequent epoxides commonly spontaneously undergoing a variety of abiotic oxidation and hydrolysis reactions (e.g. Fox *et al.*, 1990; Lontoh *et al.*, 2000). The reader is directed to Jiang *et al.* (2010), who provide an excellent summary of organic compounds attacked by both sMMO and pMMO, as well as the products of such transformation. Finally, it should be noted that not only does sMMO attack more compounds than pMMO, it turns over these compounds more quickly.

It should be stressed, however, that degradation of these compounds by either form of MMO is 'co-metabolic', i.e. the continued degradation can occur only 'in the obligate presence of a growth substrate or another transformable compound' (Dalton and Stirling, 1982) as these pollutants typically can serve neither as a carbon nor energy source to methanotrophs. Rather, transformation of these compounds requires reducing equivalents, whose consumption can limit methanotrophic growth and activity. In addition, toxic products are commonly formed from these co-metabolic reactions (e.g. Fox *et al.*, 1990; Lontoh *et al.*, 2000; Jiang *et al.*, 2010; Semrau, 2011). As a result, although use of sMMO-expressing methanotrophs for the biodegradation of organic pollutants may appear attractive given these microbes can attack a wider range of compounds and degrade them more quickly than pMMO-expressing methanotrophs, it may actually be counter-productive. That is, methanotrophs expressing pMMO, although attacking fewer co-metabolic pollutants and degrading them slower, more easily turnover methane in their presence, thus allowing for greater overall activity and growth that then leads to greater overall degradation of select pollutants. In other words, a pMMO-expressing methanotroph, or 'tortoise' may actually be better than an sMMO-expressing methanotroph, or 'hare' for bioremediation of polluted sites (Lee *et al.*, 2006). Indeed, studies have found that pMMO-expressing methanotrophs are responsible for pollutant degradation *in situ* and in enrichment cultures (Shukla *et al.*, 2009; Paszczynski *et al.*, 2011). The take-home message is that effective use of methanotrophs for pollutant degradation is not as simple as it might first seem. Rather, it requires one to consider what is to be degraded and how effective turnover of methane can be maintained to ensure that the appropriate conditions (i.e. pMMO- vs sMMO-promoting conditions) are provided to facilitate methanotrophic growth that then leads to greater pollutant removal.

Control of methane emissions

Methane is a potent greenhouse gas, with a global warming potential 84 times that of carbon dioxide over a 20-year time frame (Myhre *et al.*, 2013). The current atmospheric methane concentration of 1.8 ppm_v (or 154 ppm_v in CO_2 equivalents) is expected to double by 2100 (Saunois *et al.*, 2016). Moreover, the radiative forcing incurred per unit increase in concentration is larger for methane than for carbon dioxide (Lashof and Ahuja, 1990). Of particular concern are emissions of methane from permafrost and thermokarst lakes in warming Arctic regions (Nzotungicimpaye and Zickfeld, 2017). The positive feedback loops resulting from such releases are projected to discharge 30 to 63 gigatons of permafrost carbon to the atmosphere in the next three decades and 232 to 380 gigatons by 2100 (Schuur and Abbott, 2011). Thus, both prevention of methane emissions from warming arctic regions as well as from agriculture systems (e.g. rice paddies) is a topic of great importance, as are strategies for removal of methane from the atmosphere. Pursuing such a two-pronged approach could have a significant effect in the short term on reducing future global warming.

Currently, the primary sink of methane after entering the atmosphere is reaction with the hydroxyl radical (OH·), with initial estimates that this mechanism is responsible for ≈90% of atmospheric methane removal (Wuebbles and Hayhoe, 2002). Subsequent studies suggest this sink may be overestimated, indicating that other sinks of atmospheric methane exist (Wang *et al.*, 2008). Although microbial consumption of methane (i.e. methanotrophy) is well known to occur in the subsurface where methane concentrations are high (Hanson and Hanson, 1996), it was initially widely believed that biological removal of atmospheric methane was unlikely given the relatively poor affinity cultured methanotrophs had for methane (on the order of 1000–10,000 ppm_v). It had been speculated, however, that methanotrophs capable of oxidizing methane at atmospheric levels could exist (Bender and Conrad, 1992). It is now known that biological removal of atmospheric methane can and does occur, and that some environments, e.g. upland forest soils, act as sinks of atmospheric methane through methanotrophic activity (Bull *et al.*, 2000; Knief *et al.*, 2003; Knief and Dunfield, 2005). More recently it was shown that some methanotrophs, e.g. *Methylocystis* strain SC2, can express alternative forms of pMMO, with one isoform having an affinity for methane of ≈80 ppm_v (Baani and Liesack, 2008). Further, this methanotroph, when expressing this isoform could grow when at least 10 ppm_v methane was added and 1.75 ppm_v methane was sufficient to maintain cells.

With such information, it is now apparent that methanotrophs can oxidize methane at concentrations found in the atmosphere. Intriguingly, analyses of a forest soil exhibiting atmospheric methane uptake indicated that a member of a hitherto unknown genus in the *Beijerinckiaceae* family, with the proposed name of '*Methyloaffinis lahnbergensis*', can also carry out atmospheric methane oxidation (Pratscher *et al.*, 2018). Using the 16S rRNA and *pmoA* sequence from this genome to query available genomic databases finds that this microbe is present in a wide range of environments, including Arctic/permafrost soil, high elevation crusts, alpine grasslands as well as cave biofilms, suggesting that microbes able to take up atmospheric concentrations of methane may be more widespread than initially believed (Pratscher *et al.*, 2018).

What is equally exciting is that some locations widely believed to act primarily as methane sources (e.g. rice paddies and other agricultural soils) can, when properly stimulated, remove atmospheric methane (Ho *et al.*, 2015; Cai *et al.*, 2016). That is, in these situations, more 'conventional' methanotrophs, i.e. those that do not do not align phylogenetically with known high affinity methane oxidizers, appear to be responsible for atmospheric methane consumption, but that such consumption was dependent on periods of methane enrichment alternated with no provision of methane. Such patterns are likely to occur *in situ*, and thus it appears that different methanotrophs have developed different strategies to survive when methane approaches atmospheric concentrations. Given these findings, it may be possible to harness the activity of methane-oxidizing bacteria to not only remove methane from 'hotspots', i.e. areas where ambient concentrations are greater than 500 ppm (e.g. above landfills and in concentrated animal feeding operations; Yoon *et al.*, 2009; Ganendra *et al.*, 2015) but also directly from the atmosphere. Such strategies should be more fully examined to determine if

methanotrophs can be appropriately stimulated to remove methane from a variety of environments (including the open atmosphere) such that a net reduction of greenhouse gas emissions occurs. That is, life cycle assessments of any proposed strategy should be performed to insure that it does not indirectly lead to net greenhouse gas loading to the atmosphere, e.g. increased carbon dioxide emissions from resource and power consumption required to stimulate methanotrophic activity.

Metal detoxification

As described above, methanotrophs are sensitive to copper, and have effective means of sequestering copper from the environment. Methanotrophs, however, have been extensively shown to bind and detoxify many other metals, including toxic forms of chromium and selenium (Al Hasin *et al.*, 2010; Lai *et al.*, 2016; Eswayah *et al.*, 2017). These studies clearly show there is significant potential for the use of methanotrophy for the bioremediation of sites contaminated with these metals, but much remains to be learned as how these metals are bound, taken up and transformed. That is, although one may suspect that methanobactin may be involved in transforming these metals, it should be noted that in these studies, the methanotrophic type strain *Methylcoccus capsulatus* Bath was used, and this methanotroph does not have the operon for methanobactin. It may be that MopE and/or MopE* can bind metals such as chromium and selenium, and facilitate delivery to *M. capsulatus* Bath. Alternatively, uptake may be facilitated by some other active mechanism or it may be due to non-specific sorption onto the cell surface, e.g. possibly sulfhydryl groups (Eswayah *et al.*, 2017). In any event, detoxification is dependent on the availability of methane that likely serves as an electron source for metal reduction (Al Hasin *et al.*, 2010; Eswayah *et al.*, 2017).

Although methanobactin is not involved in metal detoxification by *M. capsulatus* Bath, methanobactin has been shown to bind and detoxify mercury. Specifically, both Group I and II methanobactins will bind Hg[II], even in the presence of copper due to the initial rapid binding of Hg[II] (Vorobev *et al.*, 2013; Baral *et al.*, 2014). Mercury binding by methanobactin also reduced Hg[II] toxicity to methanotrophs unable to produce methanobactin, suggesting that methanotrophic production of methanobactin *in situ* may provide protection to the broader microbial community from mercury toxicity (Vorobev *et al.*, 2013).

Interestingly, not only will methanobactin bind inorganic mercury, it can also bind the much more toxic methylmercury (Baral *et al.*, 2014). Interestingly, methylmercury uptake seems to be a general phenomenon exhibited by methanotrophs, including those that do not possess the methanobactin gene cluster (e.g. *M. capsulatus* Bath; Lu *et al.*, 2017). It is unknown how these methanotrophs sequester methylmercury, but again MopE or MopE* may play a role. Interestingly, however, only those methanotrophs capable of expressing methanobactin were found to not only sequester, but also degrade methylmercury (Lu *et al.*, 2017). Such a finding is remarkable as in these methanotrophs, *merB*, encoding for the organomercurial lyase, is not in their genomes, indicating that some other mechanism demethylates methylmercury. Although one may suspect that methanobactin may be responsible for both methylmercury uptake and degradation, assays using purified methanobactin found no significant methylmercury degradation, suggesting that methanobactin works in concert with (as yet unknown) proteins to facilitate methylmercury degradation. Whatever the process whereby methylmercury is degraded by methanotrophs expressing methanobactin, such findings may have significant environmental relevance as in these assays, low (nanomolar) levels of methylmercury were used, similar to that found in the environment (Barkay and Wagner-Döbler, 2005; Lu *et al.*, 2016).

Methanotrophic sequestration of copper and its impact on activity of other microbes

Although methanobactin will adventitiously bind a variety of metals in addition to copper (i.e. mercury), most metals are bound with much weaker affinity (Choi *et al.*, 2006a). Thus, the predominant (but clearly not the only possible) function of methanobactin is to collect copper to support methanotrophic physiology. Sequestration of copper by methanobactin could then prevent other microbes from collecting copper as they may not have the mechanism required for uptake of copper–methanobactin complexes and/or be able to liberate copper from methanobactin. It should

be noted that although copper can be toxic due to its high redox activity and binding to iron–sulfur cluster sites (Macomber and Imlay, 2009; Pham *et al.*, 2013), copper is required as a trace nutrient, and respiration by some microbes is strongly dependent on copper availability. For example, complete denitrification of nitrate to dinitrogen requires copper for the activity of the nitrous oxide reductase (NosZ (Brown *et al.*, 2000)). When several denitrifying bacteria were incubated axenically, little and transient amounts of N_2O were observed. When these same cultures were incubated either in the presence of methanobactin or active cultures of *M. trichosporium* OB3b, substantial amounts of N_2O were observed, indicating that methanobactin, through binding of copper, limited the activity of NosZ (Chang *et al.*, 2018). In support of this conclusion, when denitrifying bacteria were incubated in the presence of a mutant of *M. trichosporium* OB3b defective in the production of methanobactin, little N_2O was observed. It should be stressed that microbes that occupy niches similar to methanotrophs, e.g. ammonia-oxidizing bacteria and archaea as well as the methanethiol degrading methylotroph, *Hyphomicrobium* sp. VS, also have strong copper requirements (Ensign *et al.*, 1993; Juliette *et al.*, 1995; Walker *et al.*, 2010; Amin *et al.*, 2013; Jacquot *et al.*, 2014; Eyice *et al.*, 2018). It would be of great value to determine if copper competition between methanotrophs and these microbes also occurs and, if so, what the impact may be.

Medical applications of methanotrophy

Treatment of Wilson disease

Potential use of methanobactin in treating other copper-related pathologies

Methanobactin may also be useful for treating copper-associated pathologies such as Alzheimer's disease and some cancers (Folkman and Shing, 1992; Pan *et al.*, 2002; Brady *et al.*, 2014; Garber, 2015). Alzheimer's disease is associated with the presence of excess misfolded proteins resulting in neuronal-damaging plaques (Gaggelli *et al.*, 2006; Ballard *et al.*, 2011; Davies *et al.*, 2014; Gamez and Caballero, 2015). Copper increases the severity of Alzheimer's disease by not only increasing the production of misfolded proteins, but also by inhibiting export of these proteins from brain tissue, collectively leading to greater plaque formation (Singh *et al.*, 2013). It is tempting to speculate that methanobactin, given its high affinity for copper, may be useful in treating Alzheimer's disease through the removal and/or prevention of copper partitioning to brain tissue. Although such applications are certainly intriguing, it should be stressed that much more investigation is needed to verify the utility of methanobactin for treating Alzheimer's disease.

Active oncogenesis of a variety of cancers is also influenced by copper availability. That is, copper is required for serine/threonine-protein kinase B-Raf (BRAF)-driven tumorigenesis. If copper uptake is disrupted through the addition of tetrathiolmolybdate or mutations in the copper binding site of BRAF, tumorigenesis is inhibited (Brady *et al.*, 2014). As such, inhibiting kinase activity with methanobactin, a much more potent copper chelator, merits consideration for the treatment of a variety of cancers.

Industrial applications of methanotrophy

As noted earlier, methane prices have decreased significantly in recent years, largely due to the development of technologies to extract methane from previously inaccessible locations, e.g. hydraulic fracturing of shales coupled with directional drilling. This has fostered a great deal of interest in the use of methanotrophy to valorize methane (a relatively inexpensive substrate) to diverse products including protein (for use as a feed supplement) liquid fuels (for use in the transportation sector), plastic precursors (for use in packaging and biomedical applications), osmo-protectants (for use in cosmetics), and potentially as human health supplements. Here we summarize current products and strategies of methane 'bio-valorization' as there have been recently many excellent reviews on the valorization of methane via methanotrophy (e.g. Chistoserdova and Kalyuzhnaya, 2018; Fei *et al.*, 2014; Kalyuzhnaya *et al.*, 2015; Khmelenina *et al.*, 2015; Strong *et al.*, 2015, 2016, 2017; Kalyuzhnaya, 2016). The reader is strongly encouraged to peruse these reviews for more information.

Production of single-cell protein

The initial industrial application of methanotrophy for product development was the conversion of methane to protein. Commercial production first started with the company now known as UniBio (www.unibio.dk), producing 'uniprotein' using a continuous fermentation process cultivating a moderate thermophilic methanotroph, *Methylococcus capsulatus* Bath. The resulting product has been used as a feed supplement for a number of animals, including chickens, various fish species, dogs and mink (Øverland *et al.*, 2010a,b; Romarheim *et al.*, 2011; Anvar *et al.*, 2014). It should be kept in mind that axenic cultures were not possible, with the system repeatedly invaded by an *Aneurinibacillus* species, a *Brevibacillus agri* strain, and a *Ralstonia* species (Bothe *et al.*, 2002). These contaminants, however, are of limited concern as they do not produce enterotoxins. Rather their presence seems beneficial as they appear to consume metabolites that otherwise may have inhibited methanotrophic growth. Interest in this area is increasing, with a second company, Calysta, also using a methanotrophic platform for the production of single-cell protein (www.calysta.com).

Plastic production

Another current commercial application of methanotrophy is the production of polyhydroxyalkanoates for bioplastics. In particular, methanotrophs that rely on the serine cycle for carbon assimilation (primarily α-Proteobacteria methanotrophs, but some γ-Proteobacteria methanotrophs appear to have components of the serine cycle (Ward *et al.*, 2004; Semrau *et al.*, 2010)) are targeted for production of polyhydroxyalkanoates. The serine cycle can be conjoined with the ethylmalony-CoA pathway where the intermediate 3-hydroxybutryl-CoA can be converted into polyhydroxybutyrate. A great deal of research has shown that the production of polyhydroxyalkanoates is dependent on growth conditions, e.g. copper-limitation in *M. capsulatus* Bath (Choi *et al.*, 2003) or nitrogen (as ammonia) limitation in *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b (Asenjo and Suk, 1986; Rostkowski *et al.*, 2013). There is also some effort under way to generate modified polyhydroxyalkanoates through the addition of valerate or *n*-pentanol (Myung *et al.*, 2015; Cal *et al.*, 2016). A significant reason methanotrophy is attractive for the conversion of methane to polyhydroxyalkanoates is the naturally high conversion rate, i.e. up to 68% of dry biomass can be polyhydroxyalkanoates (Asenjo and Suk, 1986) although yields on the order of 50% or less are more common (Pieja *et al.*, 2011; Chidambarampadmavathy *et al.*, 2015; Myung *et al.*, 2015; Zhang *et al.*, 2017). As a result, several companies are pursuing large-scale bio-production of polyhydroxyalkanoates from methane, perhaps most notably NewLight Technologies (https://www.newlight.com/) and Mango Materials (http://mangomaterials.com/).

Biodiesel production

Methanotrophs biomass naturally has a high fraction as lipids that make up the internal cytoplasmic membranes, upwards of 20% (Khmelenina *et al.*, 2015). These membranes, either as discs stacked throughput the cell for γ-Proteobacteria or along the periphery of the cell for α-Proteobacteria, are common features as they are used to house the pMMO, and thus their production can be expected to increase with increasing copper as this increases pMMO synthesis. As such, methanotrophs could be used for the production of biodiesel from these lipids. These lipids, however, are polar phospholipids mainly composed of phosphatidylglycerol, phosphoditylcholine and phosphatidylethanolamine, with $C_{16:1}$ fatty acids for γ-Proteobacteria and $C_{18:1}$ fatty acids for α-Proteobacteria. Although the size of these fatty acids is appropriate for biodiesel production, the high heteroatom content (i.e. N and P) make downstream processing more challenging than use of nonpolar lipids (Fei *et al.*, 2014; Khmelenina *et al.*, 2015; Strong *et al.*, 2015; Strong *et al.*, 2016). Further, it has been estimated that lipid content would have to be increased to ≈35% to be economically viable (Khmelenina *et al.*, 2015). Nonetheless, the use of a non-agricultural source (methane) for biodiesel production has attraction as it avoids the food versus fuel dilemma of using agricultural sources. Finally, as noted by others (Strong *et al.*, 2015, 2016), the production of polar lipids via methanotrophy may have medical benefit as some studies show that it may lower LDL:HDL cholesterol levels (Müller *et al.*, 2004). Thus, this could be a particularly lucrative application of methanotrophy.

Ectoine biosynthesis

Some methanotrophs are extremely halophilic, and as such, require osmotic stabilizers or compatible solutes. One such stabilizer is the cyclic imino acid, ectoine, that is currently biologically produced at scale using *Halomonas elongate* DSM 2581^T (Schwibbert *et al.*, 2011). This compound, with the ability to stabilize proteins, nucleic acids and nucleic acid–protein complexes has use in the cosmetic industry as a moisturizer, and some potential medical applications also exist (Pastor *et al.*, 2010). Some halophilic methanotrophs, i.e. various *Methylomicrobium* species also produce ectoine as an osmotic stabilizer (Khmelenina *et al.*, 2000, 2015; Reshetnikov *et al.*, 2006, 2011a,b). As stressed by Strong *et al* (2016), high value applications of methanotrophy for production of lipids for medical use and ectoine for cosmetics may be required to make methanotrophic creation of other products such as fuels and plastics economically viable. That is, the simultaneous realization of multiple products from methanotrophic platforms may be necessary to ensure that methane valorization can be attractive and is pursued more vigorously in the future for many different products. Doing so will make it more likely that we can achieve the more important and broader goals of creating more sustainable production platforms while also reducing net emissions of greenhouse gases like methane.

Current/future strategies for the enhancement of methane valorization

Obviously, there is great, yet unrealized potential for the use of methanotrophy to convert methane to various valuable products. These opportunities in many (but not all) cases have yet to be commercialized for a variety of reasons, including slow methanotrophic growth, limited product yield, and low rates of production. These difficulties are compounded by the fact that methane is only sparingly soluble in water, with mass transfer limitation a major obstacle to scaling up production. One must also realize the methane–air mixtures can be explosive (i.e. the lower and higher explosive limits of methane–oxygen are 5–15% (Zlochower and Green, 2009)). Nonetheless, there are ingenious engineering strategies to circumvent many of these problems via novel reactor/gas transfer designs that are reviewed elsewhere (Fei *et al.*, 2014; Strong *et al.*, 2015, 2016). Here we will focus on efforts to manipulate methanotrophic metabolism to enhance methane valorization.

Manipulation of growth conditions

The simplest strategy for enhancing methanotrophic platforms for methane valorization is the use of selective growth conditions. Such strategies have been frequently employed, e.g. varying copper to control expression of alternative forms of MMO, enhanced polyhydroxyalkanoate production under nitrogen limitation etc. Such efforts, although relatively simple in concept, could in practice be challenging given the wide range of parameters one may wish to vary to direct methane-derived carbon to various products. One promising strategy to advance screening of various conditions would be the use of microfluidic systems where many parameters could be varied simultaneously. Although this approach has some attraction, the provision of methane and oxygen may be problematic in such systems, and a simple, yet robust screening method must be created to quantify levels of product(s) of interest easily and quickly. To the best of our knowledge, such efforts have not been discussed in the archival literature, but warrant further consideration to not only screen extant methanotrophic species for enhanced product creation, but also to isolate and characterize novel methanotrophs from different environments.

Metabolic engineering

One emerging strategy to enhance the utility of methanotrophy for methane valorization is the use of metabolic engineering to remove bottlenecks and/or engineer shunts to facilitate production of specific compounds. This is increasingly attractive, especially as genetic tools have been developed for many methanotrophs (Kalyuzhnaya *et al.*, 2015), as well as increasingly more methanotrophic genomes have been sequenced. Initial efforts have shown that methanotrophs can be engineered for the production of lactic acid and carotenoids, as well as increased production of fatty acids (Ye *et al.*, 2007; Henard *et al.*, 2016; Demidenko *et al.*, 2017). Although titres to date are low, these studies clearly show proof-of-concept and are well worth pursuing further.

Use of mixed microbial communities for methane valorization

One very intriguing future research direction is not the use of axenic cultures for methane valorization, but the deliberate construction of mixed communities. That is, although axenic cultures are critical for elucidating pathways and mechanisms of substrate transformation, artificial or synthetic communities may perform better for specific applications. That is, mixed cultures of methanotrophs with heterotrophs that remove toxic metabolites may be needed for the long-term stable activity of methanotrophs [as was concluded for the production of single cell protein (Bothe *et al.*, 2002)].

On the other hand, it may be infeasible to engineer a methanotroph to carry out the transformation of methane to any specific product as the amount of effort may simply be too great for any one species. Rather, it may be more profitable to use a 'division of labour', where different microbes perform different steps in some metabolic pathway (Tsoi *et al.*, 2018). In this case, methanotrophs could be engineered to convert methane to some intermediate that would then be further transformed by another microbe – perhaps an engineered heterotroph – to further the conversion of this intermediate to the final product. One could then envisage even grander interactions between community members, i.e. where the heterotroph, in addition to removing some of the metabolic burden from methanotrophs for methane conversion may also provide some trace nutrient to the methanotroph that stimulates the overall population. Such an idea is certainly intriguing, and the idea of microbial interdependence an important concept in microbial ecology (Hug and Co, 2018). Indeed, it has been found that methanotrophs thrive better in the presence of heterotrophs (Ho *et al.*, 2014), possibly through provision of cobalamin from the heterotrophs (Iguchi *et al.*, 2011b). As such, in developing synthetic microbial communities for methane valorization, one should consider engagement between various partners in all directions. To be sure, this is a complex problem, but advances in modelling strategies could provide insight into how best to foster specific microbial interactions (e.g. Larsen *et al.*, 2012; Xiao *et al.*, 2017).

Conclusions and suggestions for future work

As a field, methanotrophy has made significant breakthroughs for environmental applications such as pollutant degradation, and is on the cusp of making equally, if not more significant contributions for the control of greenhouse gas emissions as well as the valorization of methane. There is much that remains to be learned, however, to ensure that optimal strategies are developed. Future work should examine in more detail how to best enhance methane consumption *in situ* while minimizing impact on production of other greenhouse gases such as nitrous oxide, given that methanotrophs can 'starve' other microbes for copper. Along these lines, the exact mechanism whereby different metals are detoxified, as well as how to best manipulate methanotrophic activity *in situ* for enhanced pollutant degradation needs to be more fully explored. For methane valorization, there is still much more information to gather as how best to apply metabolic engineering/synthetic microbial systems to shunt methane-derived carbon effectively to specific products. There is also a great deal of work to be done elucidating how different forms of methanobactin are synthesized, and how one can modify either precursor polypeptide or biosynthetic enzymes to alter the size and composition of mature methanobactin. It is hoped that overviews such as this stimulate more interest in the study of methanotrophy, an intriguing group of microbes.

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