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Review

REVIEW – Physiology & Biochemistry

Methanobactin from methanotrophs: genetics, structure, function and potential applications

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One sentence summary: The state of the art of the copper-binding compound methanobactin.

Editor: Rich Boden

ABSTRACT

Aerobic methane-oxidizing bacteria of the *Alphaproteobacteria* have been found to express a novel ribosomally synthesized post-translationally modified polypeptide (RiPP) termed methanobactin (MB). The primary function of MB in these microbes appears to be for copper uptake, but MB has been shown to have multiple capabilities, including oxidase, superoxide dismutase and hydrogen peroxide reductase activities, the ability to detoxify mercury species, as well as acting as an antimicrobial agent. Herein, we describe the diversity of known MBs as well as the genetics underlying MB biosynthesis. We further propose based on bioinformatics analyses that some methanotrophs may produce novel forms of MB that have yet to be characterized. We also discuss recent findings documenting that MBs play an important role in controlling copper availability to the broader microbial community, and as a result can strongly affect the activity of microbes that require copper for important enzymatic transformations, e.g. conversion of nitrous oxide to dinitrogen. Finally, we describe procedures for the detection/purification of MB, as well as potential medical and industrial applications of this intriguing RiPP.

Keywords: methanotrophs; methanobactin; copper

INTRODUCTION

Aerobic methane oxidizers—methanotrophs—utilize methane as their sole source of carbon and energy by coupling methane oxidation to dioxygen reduction. These microbes, although having a relatively uncommon ability to oxidize methane, are actually quite widespread in nature, being found wherever methane–air interfaces develop. That is, methanotrophs have been identified in diverse locations in both terrestrial ecosystems (e.g. agricultural soils, forest soils, landfill cover soils, tundra and volcanic soils, among others) and aqueous ecosystems (e.g. in the water column and sediments of freshwater and marine environments) (Hanson and Hanson 1996; Op den Camp *et al.* 2009; Semrau, DiSpirito and Yoon 2010).

All extant aerobic methanotrophs utilize the methane monooxygenase (MMO) for conversion of methane to methanol, and evidence of these genes is a common diagnostic tool for the presence of methanotrophs in environmental samples (Stralis-Pavese *et al.* 2004; Gebert *et al.* 2008; Héry *et al.* 2008; McDonald *et al.* 2008; Han *et al.* 2009; Kumaresan *et al.* 2009, 2011a,b, 2018; Lee *et al.* 2009; Vishwakarma *et al.* 2009; Im *et al.* 2011a; Bodelier *et al.* 2013; Saidi-Mehrabad *et al.* 2013; Esson *et al.* 2016). As a result, it has been discovered that methanotrophy is phylogenetically widespread, with aerobic methanotrophs identified in the *Alpha-* and *Gammaproteobacteria* classes, as well as the Verrucomicrobia and NC10 phyla (Dunfield *et al.* 2007; Pol *et al.* 2007; Islam *et al.* 2008; Op den Camp *et al.* 2009; Ettwig *et al.* 2010; Anvar

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et al. 2014; Knief 2015; DiSpirito et al. 2016). Methanotrophs in the NC10 phylum are unique in that these microbes produce oxygen required for methane oxidation via MMO by dismutation of nitric oxide (formed from nitrite reduction) to dinitrogen and dioxygen (Ettwig et al. 2010; Zhu et al. 2019). There have been recently a number of very thorough reviews on the physiology, biochemistry and microbial ecology of methanotrophs, and in the interest of brevity, the reader is recommended to consider these for more information on these topics (Knief 2015; Chistoserdova and Kalyuzhnaya 2018; Khmelenina et al. 2018; Semrau et al. 2018; Kalyuzhnaya, Gomez and Murrell 2019).

What is germane for the discussion here is that in aerobic methanotrophy, two forms of MMO have been characterized—a cytoplasmic or soluble methane monooxygenase (sMMO; E.C. 1.14.13.25) and a membrane-associated or particulate methane monooxygenase (pMMO; E.C. 1.14.18.3). sMMOs are a member of the multicomponent soluble di-iron monooxygenases used by diverse bacteria for oxidation of both aliphatic and aromatic hydrocarbons, but to date only a fraction of extant methanotrophs have been shown to express this form of MMO. Rather, the vast majority of methanotrophs express pMMO, another multicomponent enzyme the activity of which depends on copper (Green, Prior and Dalton 1985; Leak and Dalton 1986a,b; Nguyen et al. 1994, 1996, 1998; Semrau et al. 1995). Only a handful of methanotrophs can express both forms and even fewer only express sMMO (Semrau et al. 2018; Kang, Dunfield and Semrau 2019). Intriguingly, many studies have shown that not only is pMMO activity dependent on the availability of copper, so is the relative expression of sMMO/pMMO by those microbes that can synthesize both forms of MMO (Stanley et al. 1983; Choi et al. 2003). One should note that characterization of pMMO/sMMO has largely focused on two strains of methanotrophs, *Methylosinus trichosporium* OB3b^T (of the *Alphaproteobacteria*) and *Methylococcus capsulatus* Bath (of the *Gammaproteobacteria*).

These studies, although largely limited to two strains, indicate that pMMO is a copper-dependent enzyme. The exact role of copper in pMMO, however, is still a subject of significant debate. That is, multiple active sites have been proposed for the pMMO, including sites containing one, two or three copper atoms, as well as a di-iron site (Chan et al. 2004, 2007; Martinho et al. 2007; Balasubramanian et al. 2010; Wang et al. 2017; Cao et al. 2018; Semrau et al. 2018; Ross et al. 2019). The various copper models argue that copper acts to oxidize methane while the di-iron model suggests that pMMO has an active site similar to that of sMMO. What is consistent, however, in various studies of pMMO is that copper is an integral part of pMMO composition as there are multiple copper-binding sites. Copper may either be involved in direct oxidation of methane or in shuttling electrons from an *in vivo* reductant to the di-iron site for dioxygen reduction (Choi et al. 2008).

More broadly, copper has been found to affect other aspects of methanotrophy, particularly methanotrophs in the *Alpha-* and *Gammaproteobacteria*. That is, intracytoplasmic membrane formation (where pMMO is located), synthesis of polyhydroxyalkonate (used as an endogenous source of reducing equivalents by methanotrophs), expression of methanol dehydrogenase (responsible for the conversion of methanol to formaldehyde, E.C. 1.1.2.7), as well as carbon conversion efficiency and biomass yield are strongly affected by copper availability (Leak and Dalton 1986a,b; Collins, Buchholz and Remsen 1991; Brantner et al. 1997; Choi et al. 2003; Kao et al. 2004; Semrau, DiSpirito and Yoon 2010; Pieja, Rostkowski and Criddle 2011a; Pieja, Sundstrom and Criddle 2011b; Farhan et al. 2015).

Given the strong response of many aerobic methanotrophs to copper and also that copper can easily be toxic if not carefully sequestered, e.g. through binding to iron–sulfur proteins and/or its high redox activity (Vita et al. 2015, 2016), it should not come as a great surprise that methanotrophs have been found to have rather intricate mechanisms to control copper uptake, storage and homeostasis. Among these mechanisms are novel copper storage proteins found in many methanotrophs. These proteins bind substantial amounts of copper (upward of 19 copper atoms per monomer) and appear to play a key role in controlling copper distribution *in vivo* (Vita et al. 2015, 2016; Dennison, David and Lee 2018).

Although ∼40% of methanotrophs with sequenced genomes show evidence of copper storage proteins (Dennison, David and Lee 2018), there appears to be greater variability in the mechanism(s) used by methanotrophs for copper collection from the environment. Specifically, methanotrophs have been shown to express either outer-membrane bound or secreted copper-binding polypeptides, e.g. some methanotrophs within the *Gammaproteobacteria* express MopE and MopE∗ [i.e. *Methylococcus* **o**uter membrane **p**rotein that has a tryptophan modified to a kynurenine that appears to enhance copper binding, and a secreted form of MopE, respectively (Karlsen et al. 2003; Helland et al. 2008; Ve et al. 2012)]. In addition, some Gammaproteobacteria methanotrophs secrete copper-binding compounds, but comparatively little is known about them, e.g. their composition has not been characterized (Choi et al. 2010).

Other methanotrophs within the *Alphaproteobacteria* do not appear to produce these compounds and instead synthesize methanobactin (MB), a secreted polypeptide that contains two heterocyclic rings that bind copper via an N_2S_2 ligand set (Kim et al. 2004; Choi et al. 2006a,b; Semrau et al. 2013; DiSpirito et al. 2016; Semrau et al. 2018; Semrau and DiSpirito 2019a,b). To date, no methanotroph has been found that has both copper-uptake systems, and of the two general systems (i.e. MopE/MopE∗ vs MB), much more is known about the expression, genetics and biosynthesis of MB than MopE/MopE∗. Thus, in this review, we focus on MB, but stress that there is fertile ground for research for those interested in studying other identified copper-binding compounds in methanotrophs, as well as discovering/characterizing additional copper-uptake systems methanotrophs are suspected to have (Gu, Ul Haque and Semrau 2017).

DISCOVERY AND DIVERSITY OF METHANOBACTINS

Although copper has been known since the early 1980s to strongly regulate methanotrophy—especially the expression of alternative forms of MMO—evidence for any copper-specific uptake system in these microbes was not generated until 1993. Using random mutagenesis, mutants of *Methylosinus trichosporium* OB3b^T constitutive for expression of sMMO in the absence/presence of copper were constructed, suggesting that some defect in copper uptake or sensing was created. Subsequently, evidence of a peptide-based copper-binding compound was generated (Zahn and Dispirito 1996; DiSpirito et al. 1998; Tellez et al. 1998), concluding with the purification and crystallization of methanobactin from *Msn. trichosporium* OB3b^T (Kim et al. 2004). This copper-binding compound, or chalkophore, was found to be a small modified polypeptide with two oxazolone rings each with an associated thioamide group that collectively serve to bind copper as Cu(II) (Choi et al. 2006a,b; El Ghazouani et al. 2011; Bandow et al. 2012; El Ghazouani et al. 2012; DiSpirito

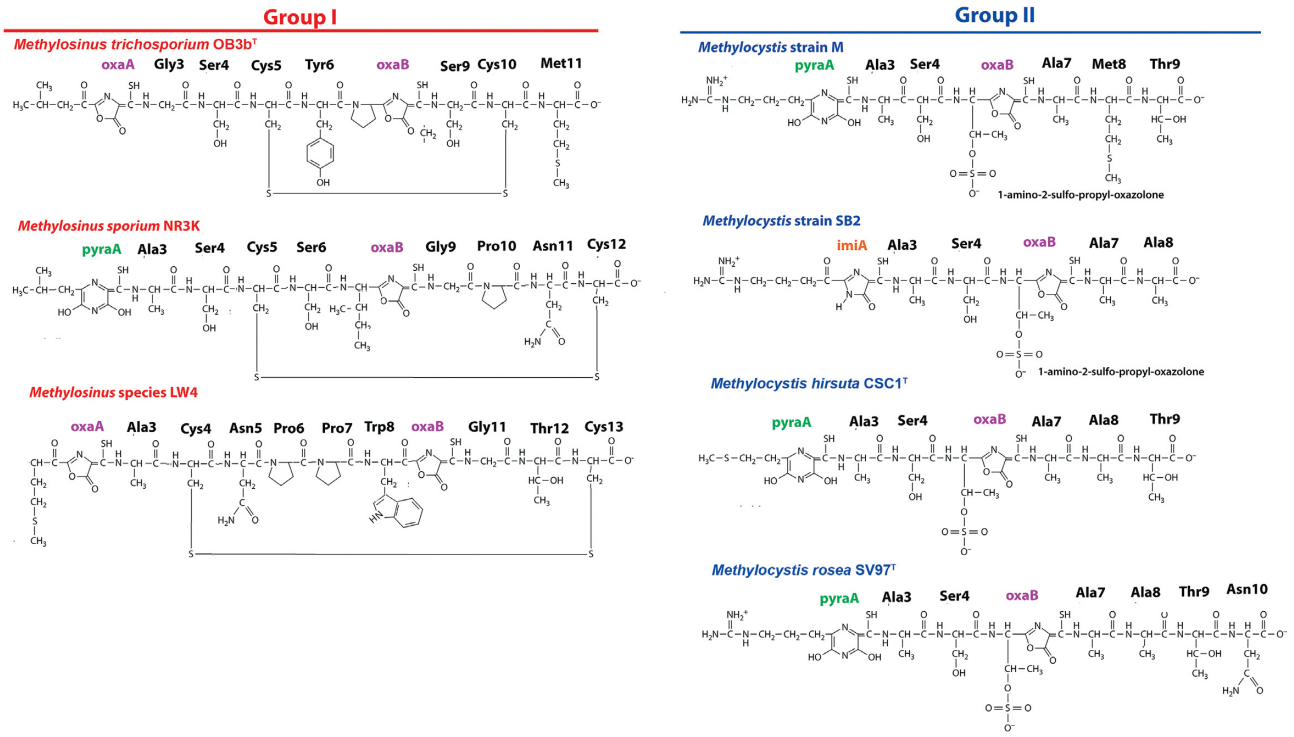

Figure 1. Primary structures of characterized Group I and II methanobactins. oxa = oxazolone; pyr = pyrazinedione; imi = imidazolone.

et al. 2016; Basle *et al.* 2018). After Cu(II) is bound, it is reduced to Cu(I) and it binds Cu(I) with high affinity (El Ghazouani et al. 2011, 2012). The reductant is as yet unidentified, although it has been speculated that water may serve as the electron source (but we stress that this has not been experimentally proven) (Krentz *et al.* 2010).

Subsequently, MBs from other methanotrophs have been purified, suggesting that MB can be categorized into two general groups: (i) Group I MBs, of which that from *Msn. trichosporium* OB3b^T was the first example, and (ii) Group II MBs, first isolated from *Methylocystis* strain SB2 (Krentz *et al.* 2010). The primary structures of all characterized MBs from methanotrophs (seven in total; three from Group I and four from Group II) are shown in Fig. 1. Although Group I and II MBs have many similarities, i.e. two heterocyclic rings with associated enethiol groups that are responsible for copper binding, there are also several significant differences. First, Group I MBs have a C-terminal oxazolone ring while the N-terminal ring is either an oxazolone ring, or in one case (for *Msn. sporium* NR3K), a pyrazinedione ring (Basle *et al.* 2018; Semrau and DiSpirito 2019a,b). Group II MBs have either an imidazolone or pyrazinedione ring as the N-terminal ring (it should be noted that these rings are tautomers), while the C-terminal ring is an oxazolone ring (Fig. 1). We would like to stress that all MBs characterized to date have a C-terminal oxazolone ring. Second, Group I MBs have an internal disulfide bridge that enables these forms to fold into a dicyclic structure after binding copper, while Group II MBs lack this bridge and form a hairpin shape after binding copper (Fig. 2) (Behling *et al.* 2008; Baral *et al.* 2014). Third, characterized Group II MBs are sulfonated (Krentz *et al.* 2010; El Ghazouani *et al.* 2012; DiSpirito *et al.* 2016, Semrau and DiSpirito 2019a,b), and such modification appears to enhance copper binding (El Ghazouani *et al.* 2012). Fourth, Group I MBs have to date only been purified/characterized from methanotrophs of the *Methylosinus* genus, while Group II MBs have only been purified/characterized from *Methylocystis* species. Bioinformatic analyses suggest, however, that some methanotrophs may be able to synthesize both forms of MB (discussed below).

GENETICS OF METHANOBACTIN BIOSYNTHESIS

Initially, it was hypothesized that due to the presence of heterocyclic rings in MB, this modified polypeptide was created via a non-ribosomal polypeptide synthase. Subsequent acid digestion assays, however, suggested that these rings may in fact be derived from a dipeptide sequence, and that one of these amino acids was likely a cysteine (Krentz *et al.* 2010), i.e. MB was more likely a ribosomally synthesized post-translationally modified polypeptide (RiPP). Scanning of the genome of *Msn. trichosporium* OB3b^T identified one gene, *mbnA*, that appeared to encode for the polypeptide precursor of mature MB. This polypeptide included both a leader and a core peptide with a potential cleavage site, indicating that the mature product was secreted. Knock-out of *mbnA* indicated that it did indeed encode for the precursor of MB as no production was observed in such a mutant (Semrau *et al.* 2013).

mbnA is part of gene cluster that includes many genes with functions either experimentally validated or presumed based on bioinformatic comparisons (Fig. 3 and Table 1). For all identified MB gene clusters in methanotrophs, immediately adjacent to *mbnA* are two genes, *mbnBC*, that appear to encode for proteins required for the formation of the C-terminal oxazolone ring from a XC dipeptide (Kenney *et al.* 2018). In addition, all identified MB gene clusters in methanotrophs have *mbnM* (believed but not proven to be responsible for MB secretion) and *mbnT* [shown to encode a TonB-dependent transporter (E.C. 7.2.2.*x*) responsible for MB uptake in at least *Msn. trichosporium* OB3b^T (Gu *et al.* 2016)].

Several other genes are also often found in MB gene clusters, but are not consistently co-located, including *mbnIR* (encoding

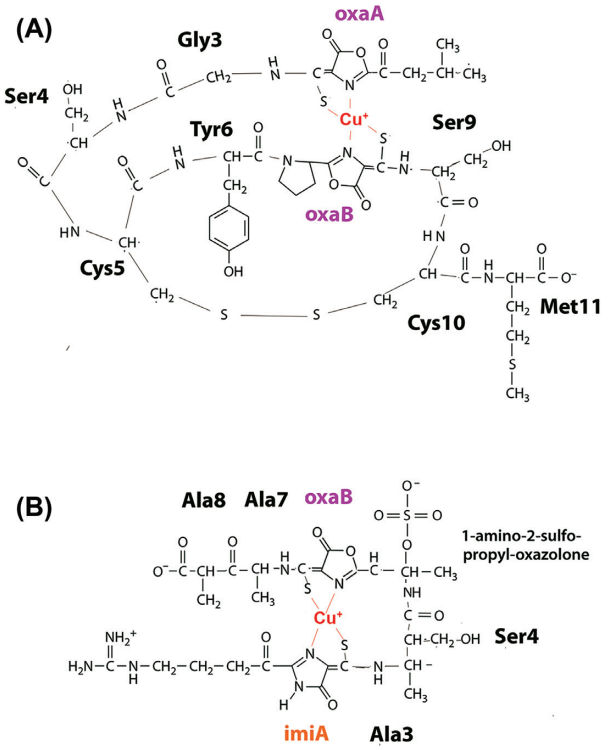

Figure 2. Structures of MB from **(A)** *Methylosinus trichosporium* OB3b^T and **(B)** *Methylocystis* sp. strain SB2 after binding copper. Note that MB from *Msn. trichosporium* OB3b^T (Group I MB) has a dicyclic structure, while MB from *Methylocystis* sp. strain SB2 (Group II MB) has a hairpin-like form when copper is bound.

for an extracytoplasmic sigma factor and membrane sensor protein, respectively) and *mbnPH* (encoding for a diheme cytochrome *c* peroxidase and its partner protein). It has been suspected that MbnIR plays a role in regulating expression of *mbn* genes via interaction with MbnT (Kenney and Rosenzweig 2013), but experimental data cast doubt on this conclusion (Gu *et al.* 2016). Thus, the role of these two genes in MB production is still an open question. Further, it has been speculated that MbnPH may play a role in the formation of the heterocyclic rings in MB and/or aid in copper release from MB (Kenney and Rosenzweig 2018), but this has yet to be examined in any detail. It should also be noted that several MB gene clusters lack *mbnPH* (Fig. 3), suggesting that these two genes are either not critical for MB production/copper release or that homologs elsewhere in the genome of various methanotrophs may serve in their place.

For Group I MBs with an N-terminal oxazolone ring (e.g. *Msn. trichosporium* OB3b^T), an aminotransferase (E.C.2.6.1.*x*) encoded by *mbnN* is critical for the formation of this ring (Gu *et al.* 2017). Not all MB gene clusters putatively encoding for a Group I MB, however, include *mbnN*, suggesting that methanotrophs with these clusters may not have an N-terminal oxazolone ring, e.g. like that found in *Msn. sporium* NR3K. Regrettably, the genome of this strain is not available (Basle *et al.* 2018), limiting our ability to make firm statements on this issue. Alternatively, homologs of *mbnN* that could serve in its place are found in methanotrophic genomes (data not shown), and it has been shown that other aminotransferases can fulfill the function of MbnN (Park *et al.* 2018).

All identified Group II MB gene clusters in methanotrophs include *mbnF*, encoding for a putative FAD-dependent oxidoreductase. It is thus tempting to speculate that *mbnF* is involved in the formation of N-terminal imidazolone/pyrazinedione ring in Group II MBs, but it must be noted that no experimental data have been published showing that this is indeed the case. It has been suggested that *mbnF* is required for the formation of the N-terminal pyrazinedione ring from an oxazolone precursor (Kenney and Rosenzweig 2018) but as *mbnN* has been shown to be necessary for formation of this ring and most Group II MB gene clusters lack *mbnN* (although methanotrophs have aminotransferases encoded elsewhere in their genome; data not shown), such a conclusion should be considered at best tentative without more explicit empirical evidence. Finally, many but not all MB gene clusters presumed to encode for Group II MBs also have *mbnS*, encoding for a putative sulfotransferase believed (but not experimentally shown) to sulfonate threonine that is combined with a cysteine to form the C-terminal oxazolone ring.

Finally, some methanotrophs appear to have multiple MB gene clusters encoding for both Group I and II MBs, i.e. *Methylocystis parvus* OBBP, *Methylocystis* sp. LW5, *Methylosinus* sp. LW3, *Methylosinus* sp. R-45379 and *Methylosinus* sav2. To date, neither expression of any of these genes nor purification of MB from any of these strains has been reported. As such, it is unknown if these methanotrophs produce either or both general forms of MB, and if so, under what conditions. It should also be stressed that *mbnIR* and *mbnPH* are not part of the several of these identified MB gene clusters; rather homologs of these genes are found some distance away. It is thus unclear (i) what role these genes have in MB production, (ii) if the lack of co-localization with *mbn* genes with clearly identified function affects the ability of these strains to produce MB or (iii) if MB made by these strains is used as a chalkophore or if it serves some other purpose as MB appears to be a 'moon-lighting' protein with multiple functions as described below.

Finally, we would like to highlight that to date, core genes for MB biosynthesis (i.e. *mbnABCM*) have only been found in the genomes of *Methylosinus* and *Methylocystis* species and that not all methanotrophs in these genera have these genes (Kang, Dunfield and Semrau 2019). Indeed, *mbn* genes are only found in ~10% of sequenced methanotrophic genomes (Dennison, David and Lee 2018). Such data indicate that MB production is a 'not' a universal trait of methanotrophs. Rather, other methanotrophs appear to rely on alternative copper-uptake systems for copper collection, or even 'steal' MB, suggesting that there may be copper competition between methanotrophs as seen between methanotrophs and denitrifying bacteria (described below in more detail). We also note that the presence of *mbn* genes is 'not' strictly correlated with the ability to express different forms of MMO, i.e. some methanotrophs with *mbn* genes can only express pMMO, while others can express both sMMO and pMMO (Kang, Dunfield and Semrau 2019).

As the majority of methanotrophs appear to not make MB, the significance of it in the environment is still an open question, although *in vitro* studies indicate that it may play a critical role in controlling copper bioavailability to the broader microbial community as discussed below (Chang *et al.* 2018). A question that then should be raised is, 'where can one expect to find MB production'? To the best of the authors' knowledge, no data have been presented documenting either the presence of MB or expression of *mbn* genes from environmental samples. As *Methylosinus/Methylocystis* species, however, have either been isolated or identified in diverse environments from metagenomic analyses [including peat bogs, landfill cover soils, groundwater, agricultural soils and forest soils, (Whittenbury, Phillips and Wilkinson 1970; Horz *et al.* 2002; Kolb 2009; Im *et al.* 2011a,b; Stein *et al.* 2011; Basiliko *et al.* 2013; Knief 2015; Leng *et al.* 2015; Smith and

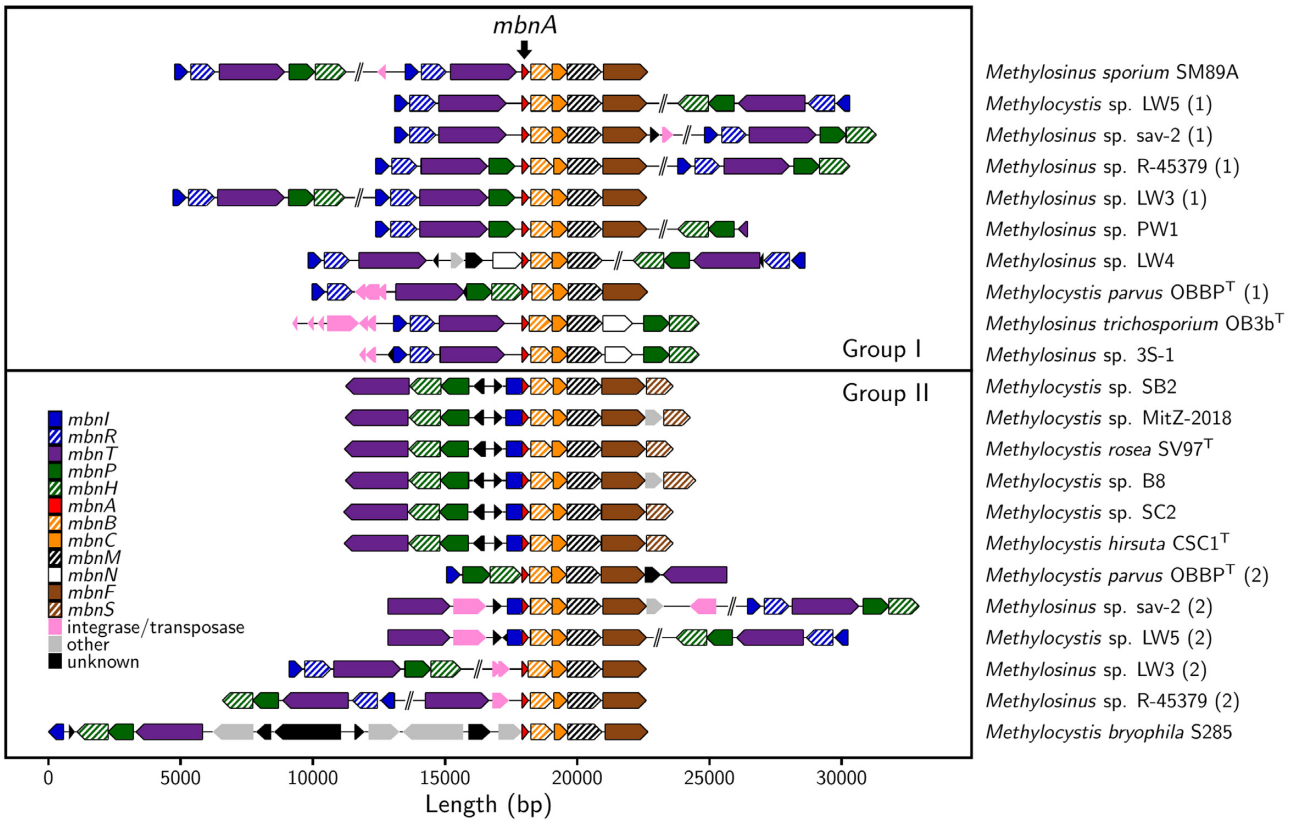

Figure 3. Identified methanobactin gene clusters from methanotrophs with available genome sequences. Four genes are found in each cluster with consistent synteny, i.e. *mbnA* (in red, encoding for the precursor polypeptide of MB); *mbnB* and *mbnC* (in shaded and solid orange, involved in ring synthesis); and *mbnM* (black shaded, believed to be responsible for MB secretion). A gene encoding for a TonB-dependent transporter (in purple; *mbnT*) responsible for MB uptake is also found in each cluster, but location varies. Other genes are also often found in MB gene clusters, but are not consistently co-located, including *mbnIR* (in shaded and solid blue, encoding for an extracytoplasmic sigma factor and membrane sensor protein, respectively) and *mbnPH* (in shaded and solid green, encoding for a putative diheme cytochrome *c* peroxidase and its partner protein). Group I MB gene clusters frequently, but not consistently include *mbnN* (in white, encoding for an aminotransferase). All Group II and many Group I MB gene clusters include *mbnF* (in solid brown, encoding for a putative FAD-dependent oxidoreductase). Several but not all Group II MB gene clusters have *mbnS* (in shaded brown, putatively encoding for a sulfotransferase).

Table 1. Known or putative role of various genes in methanobactin biosynthesis.

Gene	Known or putative function
mbnA	Precursor polypeptide of methanobactin
mbnB	Involved in synthesis of C-terminal oxazolone ring
mbnC	Involved in synthesis of C-terminal oxazolone ring
mbnM	Putative mechanism of MB secretion?
mbnP	Diheme cytochrome oxidase; may be involved in ring formation and/or copper release?
mbnH	Partner protein of *mbnP*
mbnI	Extracytoplasmic sigma factor, possible role in gene regulation?
mbnR	Membrane sensor protein, possible role in gene regulation?
mbnT	TonB-dependent transporter, required for MB uptake
mbnF	Putative FAD-dependent oxidoreductase, possible role in formation of N-terminal imidazolone/pyrazinedione ring?
mbnN	Aminotransferase, required for formation of N-terminal oxazolone ring
mbnS	Putative sulfotransferase, required for sulfonation of threonine in MB?

Wrighton 2019; Szafranek-Nakonieczna *et al.* 2019)], it may be that environmental MB production is common. We hasten to stress, however, that the presence of methanotrophs potentially capable of producing MB cannot be equated to mean that these methanotrophs have substantial activity *in situ*, and so much more remains to be done to delineate the environmental importance of MB.

EXISTENCE OF NOVEL FORMS OF MB?

What we hope is readily apparent to the reader from this discussion is that although much has been learned about MB, there are still many more questions than answers. Thus, great opportunity exists here for further research to elucidate the pathway(s) of MB biosynthesis as well as the diversity of MBs made by

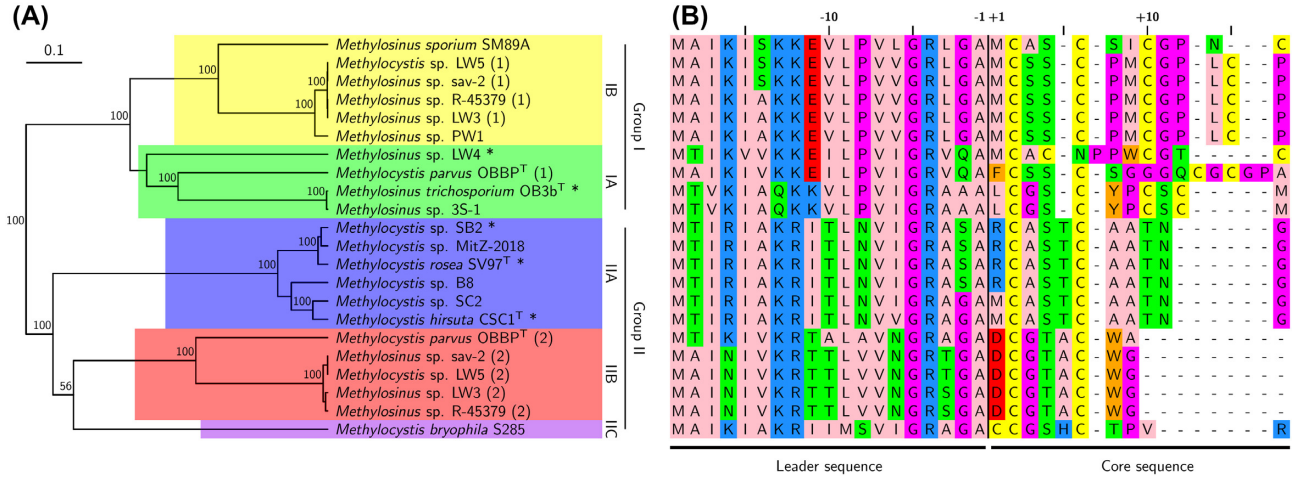

Figure 4. (A) Bayesian *mbnABCM*-based phylogeny. The tree was constructed using the general time-reversible model with invariant sites and four distinct gamma categories (GTR + I + G) under a strict clock with a minimum nucleotide sequence length of 2715. Node values indicate posterior probabilities based on 10 000 000 iterations with a burn-in of 25%. The scale bar represents changes per nucleotide position. **(B)** Predicted amino acid sequence of MbnA from corresponding methanotrophs. Note that MbnA has both a leader sequence (not observed in characterized MBs) and a core sequence (found in characterized MBs denoted by ∗).

methanotrophs. Indeed, a phylogenetic tree based on concatenated *mbnABCM* sequences (Fig. 4A; note that methanotrophs with structurally characterized MBs are identified with ∗) not only supports the original division of MBs into two general groups but also suggests these may be further divided into multiple subgroups.

Within Group I MBs, based on *mbnABCM* sequence comparison, there appear to be two subpopulations—Group IA and IB. Group IA MBs include that from *Msn. trichosporium* OB3b^{T} and *Methylosinus* sp. LW4 that have been structurally characterized. One MB gene cluster in *Mcs. parvus* OBBP and that from *Methylosinus* sp. 3S-1 are also included in this subgroup. Group IB MBs include several from a number of methanotrophs with multiple gene clusters (i.e. various *Methylosinus* and *Methylocystis* species as discussed above), as well as the *mbn* genes of *Msn. sporium* SM89A. MB of *Msn. sporium* SM89A is more distantly related in comparison with the relatively tight clustering of the rest of the group and may possibly encode for a different form of MB. It would be worthwhile to determine if these methanotrophs (particularly *Msn. sporium* SM89A) produce MBs, and if so, how structurally similar they are to known MBs. We would like to note that a partial *mbnA* gene sequence is available for *Msn. sporium* NR3K (Basle *et al.* 2018). When one compares the predicted MbnA sequence of *Msn. sporium* NR3K to that of *Msn. sporium* SM89A, there is remarkable similarity (only the first amino acid of the core peptide is different; data not shown). This suggests that different *Msn. sporium* strains may make near-identical MBs.

On the other hand, there are three general subgroups within the Group II MBs based on *mbnABCM* sequence comparison. The only identified Group II MBs are from Group IIA, i.e. various *Methylocystis* species that have significant gene synteny, phylogeny and MbnA sequences (Figs 3 and 4). One can postulate the presence of an additional subgroup (Group IIB), but MB has not been purified/characterized from any methanotrophs in this group, although it appears that both *Methylocystis* and *Methylosinus* species may produce MBs of this type. The possibility that these strains may produce a novel form of MB is supported by the fact these *mbn* gene clusters lack *mbnS*, putatively encoding for a sulfotransferase (Fig. 3). It is also interesting to note that those putative Group II MB gene clusters lacking *mbnS* appear to have the C-terminal ring of MB formed from either an Alanine-Cysteine or Histidine-Cysteine dipeptide (Fig. 4B) rather than Threonine-Cysteine as found in Group IIA MBs. The predicted core polypeptide sequence of *mbnA* from these clusters includes a threonine elsewhere, but apparently these are not sulfonated, possibly because they are not involved in ring formation. MB has not been isolated/characterized from any of these methanotrophs, and so it is unknown if these threonines are sulfonated or not, but if so, it would seem that a sulfotransferase encoded elsewhere in the genome is responsible. This appears unlikely, however, as no evidence of any gene with significant similarity to *mbnS* from *Methylocystis* strain SB2 was found in the genomes of methanotrophs putatively making Group IIB MBs (data not shown). One should keep in mind, however, that a novel sulfotransferase may be present in these genomes as a large fraction of any genome encodes for genes of unknown function.

Finally, bioinformatic interrogation of available methanotroph genomes suggests that *Methylocystis byrophila* S285 (Group IIC) may make a novel form of MB (Fig. 4A). The predicted core peptide of this putative MB has significant differences as compared to other MBs (Fig. 4B), e.g. other MBs are predicted to have either two or four cysteines in the core peptide (Group II and I MBs, respectively), but MB from *Mcs. byrophila* S285 appears to have three. This methanotroph, isolated from a sphagnum peat bog with a pH of 4.2 (Belova *et al.* 2013; Han, Dedysh and Liesack 2018), may make a modified acid-stable form of MB as other forms of MB are easily digested in dilute acid solutions, especially the oxazolone rings of *Msn. trichosporium* OB3b and *Mcs.* strain SB2 MB. We hasten to stress this is highly speculative, however, and provide these comments in the interest of stimulating further discussion and research into MB.

ROLE OF METHANOBACTIN IN COPPER UPTAKE

For those methanotrophs shown to produce MB, it is clear that it plays an important role in copper uptake, but it has also been found that methanotrophs have remarkable redundancy in their ability to sequester copper. That is, methanotrophs defective in MB production (i.e. knock-outs of either *mbnA* or the MB gene cluster from *mbnA* through *mbnN*) were still able to sense and

collect copper (Semrau *et al.* 2013; Gu, Ul Haque and Semrau 2017). Interestingly, the ability to express MB appears to amplify the cells' response to copper (Semrau *et al.* 2013).

Further, a mutant where *mbnT* encoding for a TonB-dependent transporter was knocked out showed that this mutant, although unable to take up MB, was able to sequester copper by some alternative means (Gu *et al.* 2016). If, however, exogenous MB was added to this mutant to 'soak up' all available copper, copper uptake was prevented, indicating that this transporter was indeed required for uptake of copper associated with MB. It may be that methanotrophs that express MB utilize it as a high-affinity copper-uptake system when copper is limiting, but have other, lower-affinity systems when it is not. The identity and nature of these low-affinity systems are still elusive. It has been hypothesized that *copCD,* encoding for an inner membrane and periplasmic copper-binding protein, respectively, may be involved in copper uptake (Kenney, Sadek and Rosenzweig 2016). Knock-out of these genes, either alone or in conjunction with genes for MB biosynthesis, however, does not prevent copper uptake, suggesting that they are either not involved in copper uptake, or that there are other, as yet unidentified, copper-uptake systems in methanotrophs (Gu, Ul Haque and Semrau 2017).

ALTERNATIVE FUNCTIONS OF METHANOBACTIN IN METHANOTROPHY

It has been found that MBs, in addition to strongly binding copper, have other functions, and may be considered as 'moonlighting' proteins. For example, both Group I and II MBs will strongly bind a number of other metals in addition to copper, most notably mercury as Hg(II). MB from *Msn. trichosporium* OB3b^{T} and *Mcs.* strain SB2 will both quickly and irreversibly bind Hg(II) even in the presence of copper (Vorobev *et al.* 2013; Baral *et al.* 2014). Further, Hg(II) toxicity was markedly reduced when bound by MB, suggesting that MB may have a secondary role in protecting microbes from the toxic effects of Hg(II) (Vorobev *et al.* 2013). In addition, MB enables methanotrophs to demethylate a much more toxic form of mercury, methylmercury (MeHg), despite the fact that methanotrophs found to degrade MeHg do not have *merB*, encoding for the canonical organomercurial lyase in their genome (Lu *et al.* 2017). Instead, it appears that MB acts as a mechanism to enable methanotrophs to uptake MeHg, where it is degraded by an as-yet-unknown mechanism.

As described above, MB not only binds Cu(II) but also reduces it to Cu(I) after binding Cu(II), indicating that MB is redox active. Indeed, Cu–MB complexes have been shown to have oxidase, superoxide dismutase and hydrogen peroxide reductase activities, with superoxide dismutase activity being greatest, followed by hydrogen peroxide reductase and then oxidase activity (Choi *et al.* 2008). EPR evidence also suggests that Cu–MB may serve to enhance pMMO activity by facilitating electron flow to the active site of pMMO (Choi *et al.* 2008). As such, the redox activity of MB may enable it to carry out multiple roles in methanotrophic physiology.

Finally, one of the initial characteristics identified in MB was its antimicrobial properties, i.e. it has been found to inhibit a variety of Gram-positive microbes, including vancomycin-resistant strains of *Staphylococcus aureus*, *Bacillus thuringiensis*, *Enterococcus fecalus*, as well as *Listeria monoocytogenes* (Johnson 2006; DiSpirito *et al.* 2007). Of these, the effect of copper–MB complexes has been most extensively reported against *L. monoocytogenes* (Johnson 2006), with the minimum inhibitory concentration of copper–MB on the order of 1–5 mM for this strain, with reductions in population ranging from ∼3 to 5 orders of magnitude. Further studies suggest that copper–MB inhibits respiration of *L. monoocytogenes* through an as-yet-unidentified mechanism, but possibilities include insertion of copper–MB complexes into the cytoplasmic membrane that reduces membrane integrity and/or the generation of radicals through uncontrolled electron transfer.

ENVIRONMENTAL RELEVANCE OF METHANOBACTIN IN CONTROLLING GREENHOUSE GAS EMISSIONS

Interestingly, recent data show that MB, by binding copper with high affinity, can prevent other microbes from collecting copper, i.e. MB appears to be a strategy whereby methanotrophs can 'hoard' copper for their own use, and by so doing effectively starve other microbes for this trace nutrient. For example, many enzymes require copper for their activity, including the nitrous oxide reductase (NosZ), responsible for the conversion of nitrous oxide to dinitrogen in denitrifying microbes. In the absence of MB or in the presence of a methanotrophic mutant where MB synthesis had been selectively knocked out, very little net nitrous oxide production was observed in several denitrifying microbes, indicating that conversion of nitrate to dinitrogen was unaffected, i.e. the rates of production and removal of nitrous oxide were roughly equivalent for all strains (Chang *et al.* 2018). When these denitrifiers were incubated in the presence of MB or in a methanotroph capable of synthesizing MB, however, *nosZ* expression significantly dropped while all the added nitrate was converted to nitrous oxide, i.e. nitrous oxide was not further reduced to dinitrogen due to the inability of the denitrifying bacteria to collect copper in the presence of MB. As there are many other microbes that can co-exist in the same redox environments as methanotrophs that have enzymes shown to be dependent on copper for activity [e.g. the ammonia monooxygenase of ammonia-oxidizing bacteria and archaea (Ensign, Hyman and Arp 1993; Gwak *et al.* 2019)], it is possible that methanotrophs may also limit the activity of these microbes via production of MB.

Conversely, at least some methanotrophs appear to recognize and take up MB made by others (Vorobev *et al.* 2013; Ul-Haque *et al.* 2015) suggesting that there may be, at least among some methanotrophs, MB 'theft'. Such a strategy, if occurring, is not unprecedented as there are many examples of iron 'cheaters' where some microbes 'steal' siderophores made by others to meet their metabolic needs for iron (Champomier-Vergès, Stintzi and Meyer 1996; Guan, Kanoh and Kamino 2001; Cordero *et al.* 2012; Butaitė *et al.* 2017). Thus, there appears to be a great diversity of potential interactions based on MB, and these may have significant impacts on overall microbial community activity.

POTENTIAL APPLICATIONS OF METHANOBACTIN

In addition to binding copper and mercury, MBs will also bind and reduce Au(III), and in so doing, they have been observed to produce elemental gold nanoparticles of well-defined size distributions at room temperature (Choi *et al.* 2006b; Baral *et al.* 2014). In particular, very well-defined spherical gold nanoparticles were formed with MB from *Methylocystis* sp. strain SB2, with the mean size of 2.0 ± 0.7 nm (Bandow 2014).

Such a finding is remarkable for typically biosynthesis of gold nanoparticles produces a wide range of particle sizes of varying shapes (Narayanan and Sakthivel 2010; Pourali *et al.* 2017). Further, bacterial-mediated gold nanoparticle synthesis typically involves whole cells where a variety of electron donors are available. Here, with MB, no reductant was added, and so the source of electrons for gold reduction is unclear. This issue is particularly confounding as Au(III) was not found in solution until the Au/MB ratio was greater than two, i.e. at least six electrons were transferred per MB to Au(III). As noted above, the source of electrons has not been explicitly identified, but if it is water, it appears that MB can repeatedly oxidize it by an as-yet-unknown mechanism, although such a possibility implies that metal–MB complexes, particularly an Au–MB complex, may have a very high redox potential. The resting potential of MBs is high, 550–750 mV (El Ghazouani *et al.* 2012), although the redox potential of metal-bound MB has not been determined. As speculated earlier, forcing a metal such as Cu(II) or Au(III) into a non-preferential ligand arrangement may enhance its redox potential (Krentz *et al.* 2010). Regardless of the source and mechanism of electron transfer, the finding that MB can produce gold nanoparticles indicates that this may be an alternative for their production especially as this occurred spontaneously in mild conditions at room temperature. Such a process could be advantageous as gold nanoparticles have many applications in medicine and industry, e.g. as an antimicrobial agent, fuel cell development, X-ray imaging, cancer treatment and electronics, among others (Daniel and Astruc 2004; Khlebtsov and Dykman 2010; Elahi, Kamali and Baghersad 2018).

Perhaps the most novel and meaningful application of MB, however, is its potential for use in treating copper-related diseases. That is, there are several medical conditions related to the mis-distribution of copper in the human body, most notably Wilson's disease. Individuals with this congenital disorder are unable to tolerate copper due to mutations in a specific ATPase responsible for secretion of excess copper to the bile. As a result, they are unable to expel copper from the liver, leading to copper buildup that causes severe damage. Left untreated, this disease can lead to complete liver failure as well as copper spillover into brain tissue, leading to significant neurological problems. There is no cure for Wilson's disease, and current therapies only limit future damage, i.e. they do not repair damage already incurred (Schilsky 2001; Ferenci 2005; Ala *et al.* 2007; Roberts 2011; Schilsky 2014). Wilson's disease is also considered to be an 'orphan' or 'rare' disease according to the US Orphan Drug Act of 1983, i.e. a disease with a prevalence of <200 000 people in the United States (Wilson's disease only affects roughly 1 in 30 000 people worldwide or ~10 000 people in the United States). As a result, development and testing of alternative therapies for Wilson's disease is limited given the small market, and the US Federal Drug Administration Office of Orphan Products Development actively seeks to promote development of drugs that treat it. Initial testing in rodent model indicates that MB is well tolerated and is also singularly effective in preventing copper buildup (Lichtmannegger *et al.* 2016a,b; Zischka *et al.* 2016; Müller *et al.* 2018). Further, it can also remove pre-existing copper, leading to liver repair (Lichtmannegger *et al.* 2016b). Such a finding is of particular interest as it indicates that MB may enable Wilson's disease patients to avoid having to undergo a liver transplant if severe liver damage occurs. As there are other copper-related diseases in humans [e.g. Alzheimer's Disease and BRAF-positive cancers; (Cherny *et al.* 2001; Gaggelli *et al.* 2006; Bush 2008; Squitti 2012; Brady *et al.* 2014; Brewer 2014; Squitti, Siotto and Polimanti 2014; Gamez and Caballero 2015)], there is the potential that MB may be useful for these medical conditions as well, e.g. through reducing availability of copper required for kinase activity that drives tumorigenesis in BRAF-positive cancers or limiting development of neuronal-damaging plaques in Alzheimer's disease.

METHODOLOGIES FOR MB DETECTION AND PURIFICATION

Given the importance of MB not only in controlling copper availability but also as its potential medical and industrial applications, there is a great deal of interest in screening microbes (not just methanotrophs) for MB production. To date, there are three general methodologies that have been developed. First, one can screen various available genomes for presence of genes involved in MB biosynthesis. Although one may wish to use *mbnA* as a screen, given its small size and significant sequence variability, its utility is limited. Rather, as all identified *mbn* gene clusters include *mbnB* and *mbnC*, these have often been used to screen microbial genomes for potential MB production, with both methanotrophs and non-methanotrophs shown to have MB-like genes (Krentz *et al.* 2010; Haft *et al.* 2013; Kenney and Rosenzweig 2013; Semrau *et al.* 2013). It should be stressed, however, that to date MB has only been purified from a handful of methanotrophs (Kim *et al.* 2004; Krentz *et al.* 2010; El Ghazouani *et al.* 2012; Kenney, *et al.* 2016). Although it is tempting to speculate that other microbes (including non-methanotrophs) that appear to have MB-like gene clusters do in fact produce MB, such a conclusion should be tempered by the realization that expression of these genes has yet to be determined in these cells under any condition. Are they in fact regulated by copper availability as seen in several methanotrophs? It may be that various microbes do produce some MB-like compound, but it may not be involved in copper homeostasis as MB clearly has multiple functions, e.g. controlling availability of other metals such as mercury, scavenging reactive oxygen species or acting as an antimicrobial compound. As such, it is premature to conclude what the significance of these MB-like substances may be, and that much more work is needed in this area.

Second, if one presumes that the primary role of MB expressed by any microbe is for copper uptake, then one can utilize a simple plate-based assay to determine if microbes with the putative ability to produce MB are in fact doing so. This assay, based on the popular chrome azurol S assay for siderophore production (Schwyn and Neilands 1987), substitutes copper for iron and allows for the detection of copper-binding compounds with an affinity greater than log K = 13.2 (Cha and Abruña 1990). Such an assay is useful as it is simple, economical and available in both plate and liquid versions for detection of a chalkophore (Yoon *et al.* 2010, 2011). It should, however, be considered to be a preliminary screen with more extensive isolation/purification and characterization of any putative chalkophore produced by the microbe(s) of interest then performed.

Third, procedures for isolating and purifying MB from the spent medium methanotrophs have been developed and optimized, with yields on the order of 10–100 mg per liter commonly observed (Semrau, DiSpirito and Yoon 2010; Bandow *et al.* 2011), with higher concentrations observed in the presence of limiting amounts of added copper (0.1–1 μM copper). These procedures typically require initial volumes of 1–10 liters of culture where biomass is first collected and removed (either through centrifugation or filtration) and then MB-like compound(s)

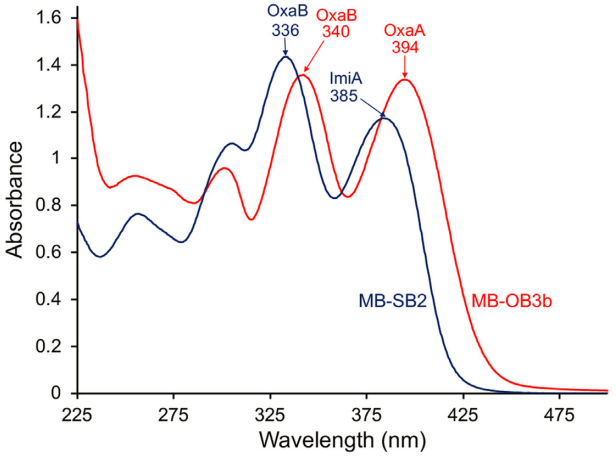

Figure 5. Characteristic UV–visible absorption spectra of Group I and Group II MBs. For Group I MB, the absorption spectra of MB from *Msn. trichosporium* OB3b^T (MB-OB3b) are shown. For Group II MB, the spectra from *Methylocystis* strain SB2 (MB-SB2) are provided.

concentrated and purified using reverse-phase chromatography. Following these procedures, MB can easily be observed via Ultraviolet-Visible (UV–Vis) absorption spectroscopy as the heterocyclic rings have characteristic absorbance peaks in the 330–340 range (for the C-terminal oxazolone group) and in the 380–400 nm range (for the N-terminal oxazolone, imidazolone or pyrazinedione group); exact wavelengths depend on the form of MB (see Fig. 5), and simple Electrospray Ionization Time-of-Flight (ESI-TOF) analyses can be used to determine the mass of collected MB.

SUMMARY

The finding and characterization of MBs in methanotrophs is the first example of a chalkophore, but as MB has been found to have multiple functions, it may serve different roles in different microbes. Interest in these novel RiPPs is increasing given their potential industrial and medical applications, as well as emerging evidence that MBs may be a critical factor regulating how microbes interact and compete for copper. Despite this, there is still more known than unknown about MB, particularly its biosynthesis, diversity and prevalence. It is strongly recommended that integrated studies of bioinformatics, genetics, biochemistry and microbial/molecular ecology be pursued to better delineate the significance of MBs, and to also enhance their utility to address a range of issues particularly important, e.g. control of greenhouse gas emissions such as nitrous oxide and treatment of grave human maladies such as Wilson's disease.

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Conflict of Interest. JDS and AAD are co-inventors in a patent application for the use of methanobactin in the treatment of Wilson's disease that has been licensed to ArborMed Company. JDS and AAD also hold a patent on methanobactin from *Methylocystis* strain SB2 (US 8629239).

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