



Metals and Methanotrophy

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ABSTRACT Aerobic methanotrophs have long been known to play a critical role in the global carbon cycle, being capable of converting methane to biomass and carbon dioxide. Interestingly, these microbes exhibit great sensitivity to copper and rare-earth elements, with the expression of key genes involved in the central pathway of methane oxidation controlled by the availability of these metals. That is, these microbes have a “copper switch” that controls the expression of alternative methane monooxygenases and a “rare-earth element switch” that controls the expression of alternative methanol dehydrogenases. Further, it has been recently shown that some methanotrophs can detoxify inorganic mercury and demethylate methylmercury; this finding is remarkable, as the canonical organomercurial lyase does not exist in these methanotrophs, indicating that a novel mechanism is involved in methylmercury demethylation. Here, we review recent findings on methanotrophic interactions with metals, with a particular focus on these metal switches and the mechanisms used by methanotrophs to bind and sequester metals.

KEYWORDS methanotrophy, methanobactin, copper, mercury, rare-earth elements

Aerobic methane-oxidizing bacteria, i.e., methanotrophs, are an intriguing group of microorganisms that utilize methane as their sole source of carbon and energy. There is great interest in these microbes, as they (i) are found wherever methane-air interfaces develop, (ii) are phylogenetically diverse, (iii) play important roles in the global carbon cycle, (iv) have been extensively used for the biodegradation of halogenated hydrocarbons, and (v) have great potential in promoting sustainability by valorizing methane to biofuels, bioplastics, and osmoprotectants, among other products (1–5).

Remarkably, aerobic methanotrophs exhibit multiple “metal switches,” where gene expression is controlled by the availability of metals, particularly copper and rare-earth elements. Further, methanotrophs have been recently shown, via the involvement of the chalkophore methanobactin, to alter the speciation and availability of other metals, including mercury. In this minireview, we provide an overview of how metals affect aerobic methanotrophy, as well as the potential applications of methanotrophy.

OVERVIEW OF METHANOTROPHY

Aerobic methanotrophs are commonly found at oxic-anoxic interfaces of terrestrial, marine, and freshwater environments where they feed on methane produced by methanogens in anoxic zones (6). Methanotrophs can also thrive in extreme environments, including those that are acidophilic, alkaliphilic, thermophilic, or psychrophilic (2, 7–14). Finally, methanotrophs have been isolated from the phyllosphere (15–18), indicating that they are widespread in nature.

Most aerobic methanotrophs group with either the *Alphaproteobacteria* or *Gamma-proteobacteria*, now with more than 20 genera combined in the two classes (Fig. 1).

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This article is dedicated to the memory of Stephen H. Stanley and Howard Dalton, leading researchers in methanotrophy who deduced the copper switch in 1983.

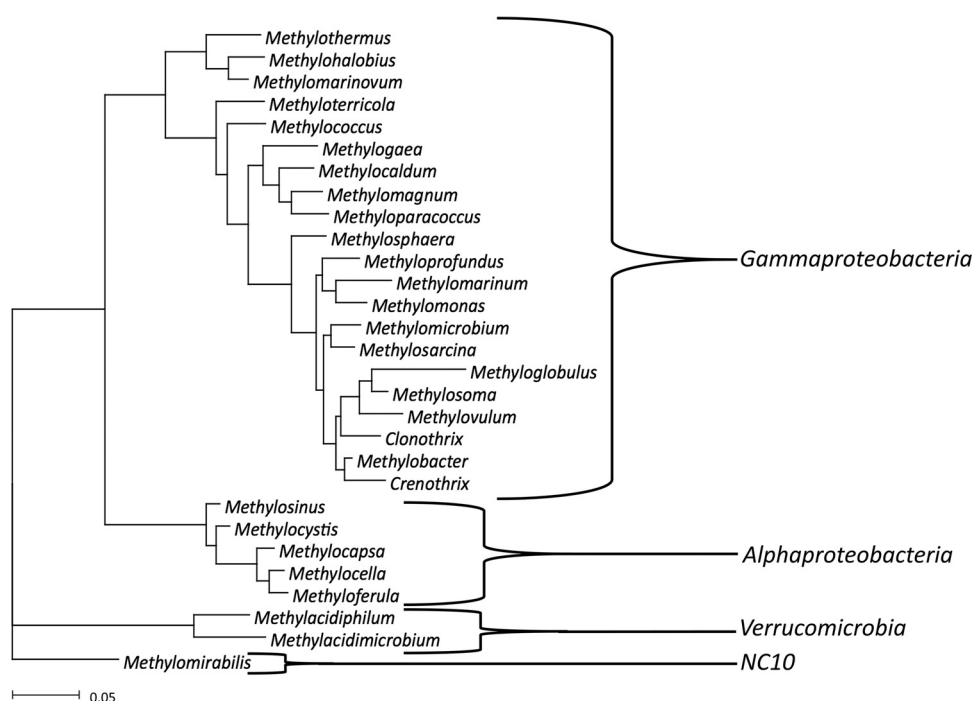


FIG 1 Phylogenetic distribution of aerobic methanotrophic genera. 16S rRNA sequence alignment was generated using the SILVA aligner (164) and used to construct a maximum likelihood tree based on the Tamura-Nei model in MEGA7 (165). For the sake of brevity, methanotrophic species are not listed. For a thorough list/description of validated aerobic methanotrophic species, the reader is directed to reference 6. It should be noted that to date, no type strains have been isolated/purified from the *Clonothrix*, *Crenothrix*, or *Methylomirabilis* genus.

Recently, however, the isolation of thermoacidophilic methanotrophs that represented distinct lineages within the *Verrucomicrobia* phylum has been reported, i.e., the *Methylacidiphilum* and *Methylacidimicrobium* genera (2, 7, 8, 11, 14, 19). Another novel methanotroph is “*Candidatus* *Methylomirabilis oxyfera*” (belonging to the bacterial NC10 phylum), which has the unique ability to couple methane oxidation to nitrite reduction, and in so doing generate dioxygen required for the initial turnover of methane to methanol (20).

Despite the broad phylogenetic and environmental distribution of aerobic methanotrophs, the pathways employed by these microbes are remarkably similar, with four general steps, as shown in Fig. 2. Methane is first converted to methanol by methane monooxygenase (MMO), which is then oxidized to formaldehyde by methanol dehydrogenase (MeDH). Formaldehyde can then be converted to formate via either the tetrahydrofolate or tetrahydromethanopterin pathway and then can be oxidized to carbon dioxide by formate dehydrogenase. Most aerobic methanotrophs take up carbon at the level of formaldehyde via either the ribulose monophosphate or serine cycle (5). Some methanotrophs, however, do not utilize either pathway for carbon assimilation. Rather, some methanotrophs fix carbon dioxide via the Calvin-Benson-Bassham (CBB) cycle, i.e., *Methyloacidiphilum fumariolicum* SolV, *Methylacidimicrobium* spp., and “*Candidatus* *Methylomirabilis oxyfera*” (19, 21, 22). Interestingly, genomes of some *Proteobacteria* methanotrophs have been found to encode the complete CBB cycle, e.g., *Methylococcus capsulatus* Bath, *Methylocapsa palsarum*, and *Methyloferula stellata* (23–25). These microbes, however, apparently cannot solely utilize the CBB pathway for carbon assimilation.

ELUCIDATION OF THE COPPER SWITCH IN METHANOTROPHS AND MECHANISM OF COPPER UPTAKE

Two distinct forms of MMO have been characterized: a cytoplasmic or soluble methane monooxygenase (sMMO) and a membrane-bound or particulate methane

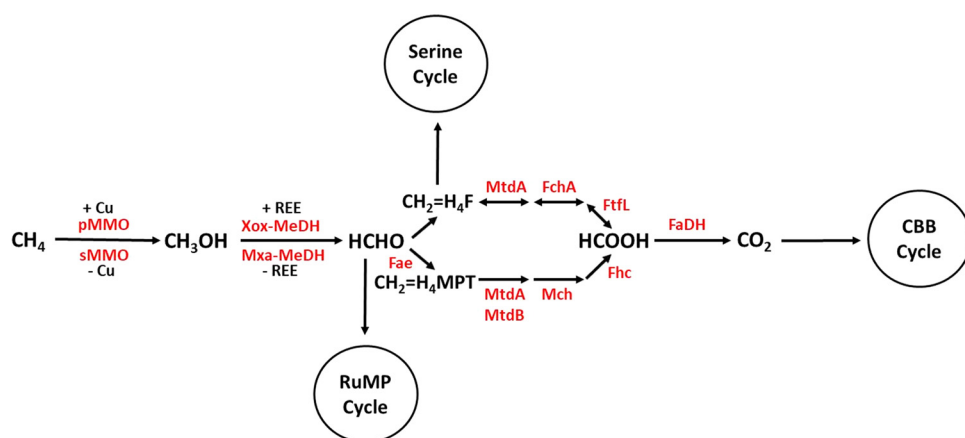


FIG 2 General pathway of methane oxidation by aerobic methanotrophs. Enzymes are noted in red. pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; Xox-MeDH, Xox-methanol dehydrogenase; Mxa-MeDH, Mxa-methanol dehydrogenase; Fae, formaldehyde-activating enzyme; H₄MPT, tetrahydro-methanopterin; MtdB, NADP-dependent methylene-H₄MPT dehydrogenase; Mch, methenyl-H₄MPT cyclohydrolase; Fhc, formyltransferase-hydrolase complex; H₄F, tetrahydrofolate; MtdA, NADP-dependent methylene-H₄F-methylene-H₄F dehydrogenase; FchA, methenyl H₄F-cyclohydrolase; FtfL, formate tetrahydrofolate ligase; FaDH, formate dehydrogenase; CBB, Calvin-Benson-Bassham; RuMP, ribulose monophosphate.

monooxygenase (pMMO). Some methanotrophs can express both forms of MMO, while others can only express pMMO, and some only have sMMO (Table 1). For those methanotrophs that can express both sMMO and pMMO, the expression of these forms is controlled by the availability of copper, i.e., the canonical “copper switch” (26). Under copper-deficient conditions, these methanotrophs will express sMMO, but as copper-to-biomass ratios increase, sMMO expression decreases substantially and pMMO expression increases. sMMO and pMMO have very different substrate ranges and kinetics; thus, it is important to understand the basis of the copper switch to exert greater control over methanotrophic activity. For example, pMMO has higher affinity for CH₄,

TABLE 1 Distribution of methane monooxygenases, methanol dehydrogenases, and methanobactin in select methanotrophs as determined via genomic interrogation for *pmoA*, *mmoX*, *mxoA*, *soxB*, and *mbnB*

Phylum or class	Family	Strain (GenBank accession no.)	pMMO	sMMO	Mxa-MeDH	Xox-MeDH	MB
Gammaproteobacteria	Methylococcaceae	<i>Methylobacter tundripaludum</i> SV96 (NZ_AEGW000000.0)	Yes	No	Yes	Yes	No
		<i>Methylocaldum szegediense</i> O-12 (NZ_ATXX000000.1)	Yes	No	Yes	Yes	No
		<i>Methylococcus capsulatus</i> Bath (NC_002977.6)	Yes	Yes	Yes	Yes	No
		<i>Methylogaea oryzae</i> JCM 16910 (NZ_BBDL000000.1)	Yes	No	Yes	Yes	No
		<i>Methyloglobulus morosus</i> KoM1 (NZ_AYLO000000.1)	Yes	No	Yes	Yes	No
		<i>Methylomarinum vadi</i> strain IT-4 (JPN0100001.1)	Yes	No	Yes	Yes	No
		<i>Methylomagnum ishizawai</i> strain 175 (FXAM000000.1)	Yes	Yes	Yes	Yes	No
		<i>Methylomicrobium album</i> BG8 (AFJF000000.2)	Yes	No	Yes	Yes	No
		<i>Methylomicrobium buryatense</i> 5G (AOTL000000.1)	Yes	Yes	Yes	Yes	No
		<i>Methylomonas</i> sp. LW13 (JNLB000000.1)	Yes	Yes	Yes	Yes	No
		<i>Methylomonas denitrificans</i> FJG1 (CP014476.1)	Yes	No	Yes	Yes	No
		<i>Methylosarcina fibrata</i> AML-C10 (ARCU000000.1)	Yes	No	Yes	Yes	No
		<i>Methyloprofundus sedimenti</i> strain WF1 (LPUF000000.1)	Yes	No	Yes	Yes	No
		<i>Methyloterricola oryzae</i> strain 73a (JYNS000000.1)	Yes	No	Yes	Yes	No
		<i>Methylovulum miyakonense</i> HT12 (AQZU000000.1)	Yes	Yes	Yes	Yes	No
		<i>Methylohalobius crimeensis</i> 10Ki (ATXB000000.1)	Yes	No	Yes	Yes	No
	Methylothermaceae						
Alphaproteobacteria	Beijerinckiaceae	<i>Methylocapsa acidiphila</i> B2 (ATYA000000.1)	Yes	No	Yes	Yes	No
		<i>Methylocella silvestris</i> BL2 (NC_011666.1)	No	Yes	Yes	Yes	No
		<i>Methyloferula stellata</i> AR4 strain AR4T (ARWA000000.1)	No	Yes	Yes	Yes	No
	Methylocystaceae	<i>Methylocystis</i> sp. strain SC2 (NC_018485.1)	Yes	No	Yes	Yes	Yes
		<i>Methylocystis</i> sp. strain LW5 (JMKQ000000.1)	Yes	Yes	Yes	Yes	Yes
		<i>Methylosinus trichosporium</i> OB3b (ADVE000000.2)	Yes	Yes	Yes	Yes	Yes
NC10	Unclassified	<i>Candidatus Methyloiridis oxyfera</i> (NSJN000000.1)	Yes	No	Yes	Yes	No
Verrucomicrobia	Methylacidiphilaceae	<i>Methylacidiphilum fumariolicum</i> SolV (CAHT000000.1)	Yes	No	No	Yes	No

while sMMO has a higher maximum CH_4 turnover rate (27). sMMO also has a wider substrate range, being able to oxidize alkanes up to C_8 , as well as ethers, cyclic alkanes, and aromatic hydrocarbons (28, 29). pMMO can oxidize alkanes up to C_5 but not aromatic compounds (29). Thus, when applying methanotrophs for the bioremediation of organic pollutants, one must carefully consider what compound(s) is (are) to be degraded. One must also consider long-term methanotrophic viability, as these pollutants typically cannot support methanotrophic growth. Methanotrophs expressing pMMO can be more advantageous than those expressing sMMO for pollutant degradation, i.e., as pMMO has greater specificity for CH_4 , it is more effective at turning over the growth substrate (methane) in the presence of nongrowth substrates (i.e., pollutants). These nongrowth substrates are still oxidized, albeit at a lower rate (27, 30). As such, for some pollutants, it is more appropriate to utilize pMMO-expressing methanotrophs, as these cultures have greater activity over time due to the continued turnover of the growth substrate, methane. Therefore, long-term bioremediation strategies using methanotrophs should carefully consider what pollutant(s) is (are) to be targeted and provide growth conditions to ensure that the appropriate MMO is being expressed.

The copper switch can be explained in part from the metal composition of the two forms of MMO. sMMO is a soluble di-iron monooxygenase and is composed of a hydroxylase, reductase, and a regulatory subunit encoded by the *mmo* operon. Methane oxidation occurs at a bis- μ -hydroxo-bridged di-iron center within the hydroxylase (31). pMMO, on the other hand, is an integral membrane metalloenzyme composed of three polypeptides arranged in an $\alpha_3\beta_3\gamma_3$ trimer (32–35) and is encoded by the *pmo* operon. There is some argument as to the active site of pMMO, with several competing models. One model proposes that methane oxidation occurs at a di-copper site in PmoB (the α subunit of pMMO [36]). Abiotic synthesis of a high-valent bis(μ -oxide) di-copper (III) complex structurally similar to the proposed di-copper site indicates that it may indeed be capable of oxidizing C-H bonds, with bond dissociation energies on the order of $75 \text{ kcal} \cdot \text{mol}^{-1}$ (37), but this is less than the bond dissociation energy of the C-H bond in methane ($104 \text{ kcal} \cdot \text{mol}^{-1}$). Alternatively, density functional theory (DFT) and quantum mechanic/molecular mechanic (QM/MM) analyses suggest that mixed-valent bis(μ -oxo) Cu(II)Cu(III) and (μ -oxo)(μ -hydroxo) Cu(II)Cu(III) complexes could abstract a hydrogen from methane (38). Although Cu(III) has yet to be observed in a metalloenzyme, Cu(III)O_2 complexes are common in synthetic chemistry (39) suggesting that it may be biologically possible. Residues for the di-copper site model, however, are not found in some pMMO sequences, e.g., verrucomicrobial pMMOs (40), and acetylene, a well-known suicide inhibitor of pMMO labels PmoA, not PmoB (41), suggesting that methane oxidation may not occur at this site. Another model proposes that the active site in pMMO is a tricopper $\text{Cu(II)Cu(II)(}\mu\text{-O)}_2\text{Cu(III)}$ complex site coordinated by residues from PmoA and PmoC (the β - and γ -subunits, respectively [42]). In support of this hypothesis, the proponents of this model show that model tricopper complexes can oxidize methane to methanol (43). A third model, based on Mössbauer spectroscopy coupled with activity measurements and metal analyses of purified pMMO, suggests that a di-iron site, similar to that found in sMMO and coordinated by residues from PmoA and PmoC, is responsible for methane oxidation (44). In support of this argument, it has been found that mutations of the predicted ligands of this site in the very similar membrane-bound hydrocarbon monooxygenase of *Mycobacterium* NBB4 abolished its activity (40).

Despite the dispute as to the exact composition of the active site of pMMO, it is generally agreed that copper is an essential component of the enzyme, as there is an additional metal-binding site in PmoB that contains a single copper (45–52). In fact, some evidence suggests that this may play an important role in methane oxidation, e.g., DFT and QM/MM studies indicate that dioxygen could be incorporated with Cu(I) to ultimately create a Cu(III)-oxo species that could convert methane to methanol (53). Further, single copper sites have been shown capable of oxidatively cleaving glycosidic bonds (54, 55), suggesting that a similar mechanism could be used to oxidize methane.

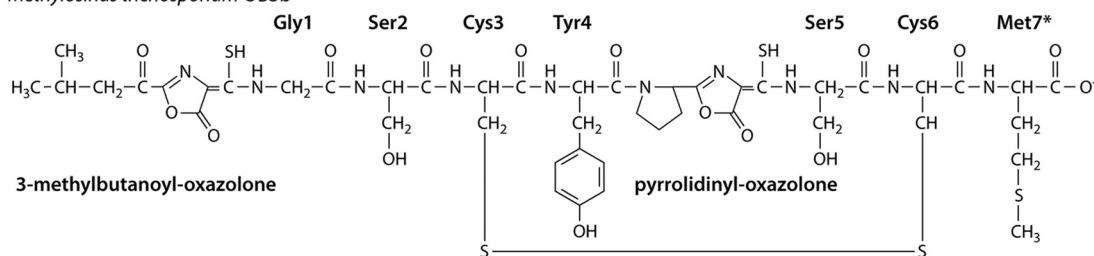
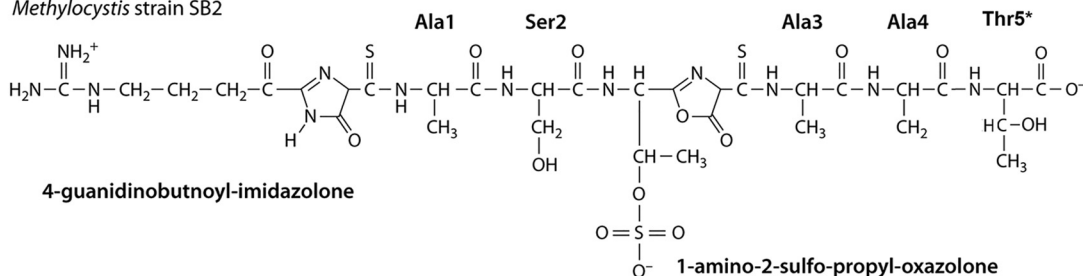
A. *Methylosinus trichosporium* OB3bB. *Methylocystis* strain SB2

FIG 3 Representative primary structures of group I (A) and group II (B) MBs. MBs from *M. trichosporium* OB3b (A) and *Methylocystis* strain SB2 (B) are shown (65, 166). *, Met7 and Thr5 are absent in a fraction of MB from *M. trichosporium* OB3b and *Methylocystis* strain SB2, respectively (59, 167). Loss of these amino acids does not change metal-binding affinity.

Given the potential widespread industrial application of a biomimetic catalyst capable of converting methane to methanol under ambient pressures and temperatures, there is a great deal of interest in delineating how pMMO oxidizes methane. It is clear, however, that more work must be pursued in characterizing this intriguing enzyme to achieve that goal.

Characterization of methanobactin, a novel copper binding molecule, or chalkophore. Methanotrophs clearly respond to copper, and as such, these microbes must have some mechanism to sense and collect copper from the environment. Initial evidence for such machinery came from the characterization of constitutive sMMO mutants (sMMO^C) of *Methylosinus trichosporium* OB3b constructed using random chemical mutagenesis (56, 57). These mutants had impaired copper uptake compared to the *M. trichosporium* OB3b wild type, suggesting that methanotrophs can synthesize a copper-complexing agent, or chalkophore ("chalko" is Greek for "copper"). Subsequently, this chalkophore was isolated, purified, and characterized from *M. trichosporium* OB3b (58). This chalkophore, called methanobactin (MB), was found to be a modified polypeptide containing two oxazolone rings with associated thioamide groups that collectively are responsible for copper binding with extremely high affinity (reported binding constants range from 10^{18} to 10^{58} M⁻¹ and vary depending on the solution conditions and method used to determine affinity [59–64]). Subsequently, MBs have been characterized from five other methanotrophs, all grouping in the *Alphaproteobacteria* (59, 65, 66). All these forms are small (<1,300 Da) but can be divided into two general groups (Fig. 3). Group I MBs have two oxazolone groups, while those in group II have a C-terminal oxazolone ring, with the other ring being either an imidazolone or a pyrazinedione moiety. Group I MBs contain Cys residues in the mature peptide, while those in group II do not. Additionally, a sulfate group is not found in group I MBs but is present in all structurally characterized group II MBs (59, 64, 65).

Biosynthesis and genetics of methanobactin. Given the unique structure of MB, it was initially speculated that it was synthesized via a nonribosomal peptide synthase (67). However, our subsequent work showed that it was a ribosomally synthesized and posttranslationally modified peptide, with the gene encoding the precursor polypeptide, *mbnA*, part of a cluster that includes genes either known or suspected to be involved in MB regulation, synthesis, secretion or uptake (Fig. 4). Upstream of *mbnA* is

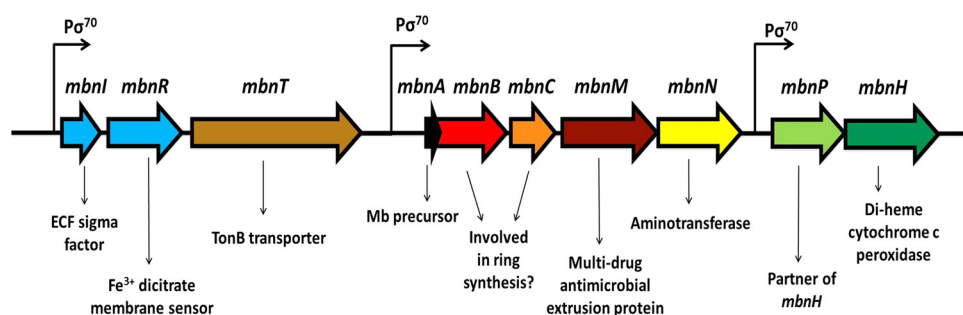


FIG 4 Methanobactin gene cluster from *M. trichosporium* OB3b. ECF, extracytoplasmic function.

mbnT, encoding a TonB-dependent transporter that we have shown is responsible for MB uptake (68). Further upstream are *mbnR* and *mbnI*, encoding a putative membrane sensor and an extracytoplasmic function sigma factor, respectively, that might play a regulatory role in MB biosynthesis. Downstream of *mbnA* in *M. trichosporium* OB3b are several other genes (*mbnBCMNPH*) that are known or suspected to be involved in the biosynthesis of MB from the precursor peptide or in MB secretion.

The sequence of *mbnB* suggests both an *S*-adenosyl-L-methionine-dependent methyltransferase and a triphosphate isomerase (TIM)-barrel domain (as predicted by Phyre2 [69]). This suggests that MbnB may have lyase, hydrolase, methyltransferase, and/or isomerase activity (70, 71). The sequence of *mbnC* includes the ligase domain of phosphoribosylformylglycinamide synthase, as well as a flavodoxin-like fold (72). Consistent with this homology, *mbnC* may be required for the formation of one or both of the heterocyclic rings. *mbnM* encodes a multidrug and toxin extrusion (MATE) protein and may be responsible for MB secretion (64, 65, 73, 74). *mbnN* is annotated as an aminotransferase that we have shown is critical for the formation of one of the two oxazolone rings in MB of *M. trichosporium* OB3b (75). *mbnH* encodes a MauG type di-heme cytochrome *c* peroxidase. In methylotrophic bacteria capable of growth on methylamine, MauG is required for the oxidative modification of two tryptophans into the cofactor tryptophan tryptophylquinone found in methylamine dehydrogenase (76–80). No methylamine dehydrogenase-like gene is present in the *M. trichosporium* OB3b genome (data not shown). Given this and the location of MbnH in the MB-OB3b operon, it appears that MbnH may be involved in oxidative step(s) required for ring formation (64). Its partner protein, MbnP, is a metallo-mystery 4-Cys motif protein.

It is difficult to predict using *mbnA* sequences if any particular microbe has the potential to produce MB; rather, interrogation of genomes for *mbnBC* is much more informative. Using these genes as markers (although, to date, only five forms of MB have been structurally characterized), bioinformatic analyses indicate that other methanotrophs in the *Alphaproteobacteria* as well as some nonmethanotrophs have genes for MB biosynthesis (Table 1) (65, 73, 74, 81). Such findings suggest that MB production may be more extensive than currently known.

Genetic basis for the copper switch. The *mmo* operon is composed of six genes, *mmoXYBZDC*. Of these genes, *mmoXYBZC* are known to encode peptides of hydroxylase, reductase, and regulatory protein, but the function of *mmoD* was unknown for many years. Characterization of the *sMMO* minus deletion mutant (SMDM), where the *mmoXYBZDC* genes were knocked out in *M. trichosporium* OB3b (82), found that the copper switch was inverted compared to the wild type (74). It was thus proposed that MmoD plays a critical role in the copper switch. This conclusion was further supported by others that found an *mmoD* deletion mutant in *Methylomicrobium buryatense* had no sMMO activity in the absence of copper (83). It should be stressed, however, that although MmoD plays an important role in the copper switch, it is likely that additional elements exist (64, 74). For example, it appears that MB serves to amplify the magnitude of the copper switch (74). Further, two genes, *mmoR* and *mmoG*, have been shown to play key roles in the regulation of expression of the *mmo* operon and that some sort

of complex may be formed between the products of these genes with methanobactin and/or MmoD. It is also possible that the putative regulatory genes associated with methanobactin, *mbnI* and *mbnR*, may play a role in the copper switch. These hypotheses must be treated cautiously, however, as no experimental data have been presented to support them.

Whole-cell transcriptome response to copper. Recently, the transcriptome of *M. trichosporium* OB3b was characterized in the presence and absence of copper with the objective of determining how broadly copper affected gene expression in methanotrophs, and of identifying additional elements of the copper switch (84). When *M. trichosporium* OB3b was grown in the presence of 10 μ M copper, approximately 100 genes were found to be either significantly up- or downregulated compared to when no copper was added. As expected, this included genes encoding polypeptides of sMMO and pMMO, as well as several putative transcriptional regulators that were significantly repressed in the presence of copper. We speculate that these putative regulatory elements may be involved in the copper switch, but this has yet to be shown.

Other genes were also found to be differentially expressed with respect to copper, including many genes involved in copper homeostasis, indicating that methanotrophs must carefully control copper speciation and distribution to limit the toxicity of copper. For example, it was found that in *M. trichosporium* OB3b, the expression of genes involved in MB transport and synthesis were repressed when copper was present (84). Additionally, two genes encoding recently identified copper storage proteins, Csp1 and Csp2 (85, 86), as well as *cusA*, encoding a copper efflux system (87), were significantly upregulated in the presence of copper. Such findings indicate careful regulation of copper uptake by *M. trichosporium* OB3b (84). Similar results have been reported for *M. capsulatus* Bath, with differential expression of *c*-type cytochromes associated with the cell surface observed, suggesting that these may play a role in copper homeostasis for this methanotroph (88, 89). Thus, although copper is very important in methanotrophic metabolism, its speciation and distribution must be carefully controlled to limit its inherent toxicity, e.g., its high redox activity and binding to iron-sulfur cluster sites (90, 91).

IMPLICATIONS OF METHANOBACTIN-METAL INTERACTIONS

Microbial competition for copper is regulated by methanobactin. Given that methanobactin binds copper with extraordinarily high affinity, Chang et al. (92) hypothesized that methanotrophs, through the production of methanobactin, could effectively “starve” other microbes for copper. Specifically, denitrifying microbes also require substantial amounts of copper for the activity of the nitrous oxide reductase NosZ (93). If this hypothesis is correct, denitrifiers when incubated in the presence of MB would not completely reduce nitrate to dinitrogen; rather, nitrous oxide would be the terminal product. Indeed, when axenic cultures of the denitrifier *Pseudomonas stutzeri* DCP-Ps1 were examined, very small and transient amounts of N_2O were observed ($\sim 0.01\%$ of added NO_3^-). When *P. stutzeri* DCP-Ps1 was incubated either in the presence of *M. trichosporium* OB3b or purified MB from this methanotroph (MB-OB3b), all added NO_3^- was converted to N_2O , with no further reduction (92). Further, when *P. stutzeri* DCP-Ps1 was incubated in the presence of a mutant of *M. trichosporium* OB3b incapable of expressing MB, N_2O production trends were identical to those of axenic cultures of *P. stutzeri* DCP-Ps1. Similar findings were found with three other denitrifiers that express NosZ (92). As methanotrophs and denitrifiers can and do spatially overlap in the environment, these findings indicate that competition for copper may significantly affect net greenhouse gas emissions *in situ*. That is, the substrates for aerobic methanotrophs are CH_4 and O_2 that diffuse toward the oxic-anoxic interface from opposite ends of the oxygen gradient. As a result, the largest abundance of methanotrophs is often found at the oxic-anoxic interfaces in subsurface environments (94, 95) that are also often a “hot spot” for soil denitrification (96).

In addition, copper competition between methanotrophs and other microbes may limit the activity of other copper-dependent enzymes, e.g., the nitrite reductase NirK and ammonia monooxygenase (93, 97). Indeed, it has been found that methanobactin can inhibit the activity of NirK in *Shewanella loihica* (92). Additional work in this area is clearly warranted.

Methanobactin can bind and detoxify mercury. Methanobactin from *M. trichosporium* OB3b (MB-OB3b) and *Methylocystis* strain SB2 (MB-SB2) will bind, in addition to Cu(II), Hg(II), and do so irreversibly, even in the presence of copper (98, 99). Such irreversible binding appears to be due to the rapid binding of Hg(II), i.e., when we attempted to measure the kinetics of Hg(II) binding by MB-OB3b, over 90% of the Hg(II) added was bound during the 1.8-ms dead time of the stopped flow reactor, and the remaining 10% had an observed binding rate of $640 \pm 43 \cdot s^{-1}$ (98). Not only was Hg(II) found to be quickly and irreversibly bound by MB-OB3b, it substantially reduced the toxicity of Hg(II) to *M. trichosporium* OB3b, as well as to other methanotrophs; i.e., methanobactin can act as a general prophylactic and protect the broader microbial community from the toxic effects of mercury. In the case of MB-SB2, Hg(II) binding rates were $>2,000 \cdot s^{-1}$, and Hg(II) could displace Cu(I) from the MB-SB2-Cu complex (99).

We have also shown methanobactin can bind methylmercury (99), suggesting that methanobactin can also control its bioavailability. Indeed, we have found that *M. trichosporium* OB3b takes up and degrades methylmercury (100). This is surprising, as *merB*, encoding organomercurial lyase, is not present in the genome of *M. trichosporium* OB3b, indicating that this strain uses another mechanism to demethylate methylmercury. It should also be noted that *M. trichosporium* OB3b degraded methylmercury at concentrations commonly found in the environment, e.g., nanomolar concentrations (100). *MerB* has a much weaker affinity for methylmercury (K_m of 500 μM) (101), and as a result, methanotroph-mediated methylmercury degradation may have great environmental significance.

Not all methanotrophs, however, can degrade methylmercury. Specifically, *Methylococcus capsulatus* Bath was found to sorb substantial amounts of methylmercury, but it did not degrade it. Genes encoding methanobactin biosynthesis are not found in *M. capsulatus* Bath. Rather, *M. capsulatus* Bath relies on a surface-bound protein with a tryptophan converted to a kynurenine (MopE) and a secreted form of it (MopE*) for copper binding (102–105). It thus appears that methylmercury degradation by methanotrophs requires that the cells first express methanobactin. This hypothesis was tested by examining methylmercury degradation by *Methylocystis* strain SB2 (capable of expressing MB) as well as two mutant strains of *M. trichosporium* OB3b defective in methanobactin production. *Methylocystis* strain SB2 degraded methylmercury, whereas MB-defective strains did not, showing that MB is required for methanotrophic-mediated methylmercury degradation. Methanobactin, although necessary, is not sufficient for methylmercury degradation by methanotrophs. When methylmercury was incubated with purified MB-OB3b, no appreciable methylmercury degradation was observed (100). This suggests that methanobactin served as a means of bringing methylmercury into the cell, where it is degraded by an as-yet-unknown mechanism.

FUNCTION OF RARE-EARTH ELEMENTS IN METHANOTROPHS

It is well known that microbial physiology requires a broad suite of metals, but some metals historically have been viewed as having little if any biological relevance. Prime examples of such metals are the rare-earth elements. These metals, despite being characterized as rare, are actually quite abundant in Earth's crust, e.g., the average abundance of cerium is $66 \mu g \cdot g^{-1}$, similar to that for zinc and copper (106). These elements, however, are typically found in extremely insoluble forms, e.g., oxides, sulfides, carbonates, and phosphates (107). Given their poor solubility, the lack of any identified biological mechanisms for uptake, and the fact that they had no established biological function, it was commonly presumed that these elements were nonessential. Despite this prevailing dogma, studies from as early as 1961 showed that some microbes could sequester large amounts of some of these metals (108). Further, it was speculated over

10 years ago that enzymes containing rare-earth elements would be catalytically superior to enzymes containing chemically similar calcium, as rare-earth elements are much stronger Lewis acids than calcium and thus are likely much more efficient catalysts for hydrolysis (109).

REEs and methanol dehydrogenase. Methanol oxidation is carried out by methanol dehydrogenase (MeDH). Initial characterization of MeDH found it to be a heterotetramer composed of two subunits, the large subunit MxaF (65 kDa) and the small subunit MxaI (8.5 kDa). MxaF contains pyrroloquinoline quinone (PQQ) and Ca^{2+} as catalytic cofactors (110–113). Subsequently, a homolog to *mxoF*, termed *xoxF*, was found in the methylotrophs *Paracoccus denitrificans* and *Methylobacterium extorquens* AM1 (114, 115). A sequence comparison revealed that most amino acid residues involved in PQQ and calcium binding in *mxoF* were conserved in *xoxF*, indicating that it was likely involved in methanol oxidation (114, 116). Early mutagenesis studies of *xoxF* in methylotrophs, however, showed either no effect or some reduction in growth on methanol (115, 116), presumably due to the coexistence of *mxoF* in the microbes examined. The deletion of *xoxF* in *Rhodobacter sphaeroides*, however, caused the resulting mutant to be unable to grow on methanol, as *R. sphaeroides* does not have *mxoF*, showing that Xox-MeDH is a true MeDH (117).

Despite this clear finding, characterization of Xox-MeDH was initially quite challenging, as its expression was typically very low, and active preparations were difficult, if not impossible, to create (118, 119). A critical breakthrough in the characterization of Xox-MeDH came from the observation that its activity was dependent on the presence of REEs (120–123). Specifically, Xox-MeDH activity increased ~5- to 10-fold with addition of lanthanum in the growth medium of the methylotrophs *Methylobacterium radiotolerans* and *Methylobacterium extorquens* AM1 (120, 122). Further, purified Xox-MeDH from *M. extorquens* AM1 was found to predominantly contain lanthanum (122). Subsequently, it was discovered that growth of the acidophilic methanotroph *Methylococcus fumariolicum* SolV, which can express Xox-MeDH but not Mxa-MeDH, was strictly dependent on the addition of REEs, with growth stimulated the greatest with “light” REEs (i.e., lanthanum, cerium, praseodymium, and neodymium). As found in *M. extorquens* AM1, purified Xox-MeDH from *M. fumariolicum* SolV also contained a rare-earth element (123).

In rapid succession, a number of papers documented that the expression of Mxa-MeDH and Xox-MeDH in a variety of methanotrophs and methylotrophs was strongly dependent on the availability of REEs, primarily light REEs (124–128). In the absence of REEs, little expression of *xox* genes was observed, while the expression of *mxo* genes was high. In the presence of REEs, however, the expression of *xox* genes increased significantly, while the expression of *mxo* genes decreased. That is, in addition to the copper switch controlling the expression of pMMO/sMMO, there is an “REE switch” regulating the expression of Mxa-MeDH/Xox-MeDH. These switches appear to have some overlap, with the copper switch capable of “overriding” part of the REE switch in at least one methanotroph. Specifically, when *M. trichosporium* OB3b was grown in the presence of copper, little repression of *mxoF* expression by REEs was observed, although substantial (>2 orders of magnitude) repression was observed when REEs were added in the absence of copper. The expression of *xoxF*, however, was not significantly different when REEs were added in the absence or presence of copper (125).

Microbial uptake of REEs. Although REEs clearly are taken up (as evidenced by the response of many methanotrophs and methylotrophs to these metals), the mechanism(s) is (are) unknown. Such uptake is particularly challenging, for as noted earlier, REEs are noted by their very low dissolution in water, e.g., CePO_4 has a solubility of 10^{-23} g per 100 g of water (129). Nonetheless, there are several possibilities as to how REEs are collected: (i) adventitious leaching of REEs via secretion of low-molecular-weight organic acids, inorganic acids, and/or metal-binding compounds, such as siderophores or chalkophores that dissolve cerium-containing minerals (130–137); (ii) secretion of a specific REE-binding compound; and/or (iii) uptake of rare-earth elements

as REE-phosphates via systems, such as the P_i transport system (Pit). This low-affinity high-velocity system is used by many microbes for phosphate uptake and has been shown to control the accumulation of metals, such as zinc (138–140). None of these possibilities can be excluded at this time. Methanotrophs are known to produce both chalkophores and siderophores (141), and these may be partially responsible for increasing the bioavailability of cerium. Further, it was discovered over 40 years ago that methanotrophs produce a number of water-soluble pigments that may include novel metal-binding compounds (142). Methanotrophs have also been shown to produce low-molecular-weight organic acids under microaerobic conditions (143), suggesting that this may play a role in the leaching of rare-earth elements. Finally, a preliminary review of the genome of *M. trichosporium* OB3b indicates that this strain does indeed have the P_i transport system (data not shown). It may be that one system or some combination of these systems is used for rare-earth element collection by methanotrophs. As REEs are difficult to purify, elucidation of the mechanism by which methanotrophs take up rare-earth elements may have significant industrial application (144).

Genetic basis of the REE switch. There is a great deal yet to be learned about the basis of the REE switch, but work from the University of Washington has begun to address this issue, i.e., Chu et al. (145) found that a histidine kinase, MxaY, is a key part of the REE switch. The deletion of *mxoY* abolishes the response of *mxoF* and *xoxF* in *M. burkatense* to lanthanum. It further appears that MxaY controls the expression of the *mxo* and *xox* genes by at least partially controlling expression of another response regulator, MxaB, although it is still unknown if MxaY and MxaB directly interact or if there are other components to the REE switch.

To identify what, if any, other elements make up the REE switch, the transcriptome of *M. trichosporium* OB3b was characterized in the presence and absence of cerium (84). Using stringent cutoff criteria of a $|\log_2|$ -fold change of >1.5 and a Benjamini-Hochberg-adjusted P value of $<1 \times 10^{-3}$, one putative sigma factor was found to be upregulated in the presence of cerium, suggesting that it may also play a part in the REE switch (84). Interestingly, when using these strict criteria, *mxoY* and *mxoB* expression was not found to vary with the presence of cerium. If, however, a more relaxed (but still stringent) criteria of a $|\log_2|$ -fold change of >1 and a Benjamini-Hochberg-adjusted P value of $<1 \times 10^{-3}$ were used, *mxoY* and *mxoB* expression was found to be repressed when cerium was added, supporting the conclusion of Chu et al. (145).

Interestingly, although the expression of Mxa-MeDH and Xox-MeDH in methanotrophs is strongly controlled by the availability of REEs, it appears that the REE switch is limited to these forms of MeDH. That is, in a comparison of the transcriptome of *M. trichosporium* OB3b grown in the presence of 25 μ M cerium versus 0 μ M cerium, the expression of only a small number of genes not involved in either Mxa-MeDH or Xox-MeDH had differential expression (84). Perhaps most interesting was that the expression of genes involved in the conversion of formaldehyde to formate via the tetrahydromethanopterin and tetrahydrofolate pathways was not affected by the presence or absence of cerium. Such a finding is intriguing, as it has been shown that Xox-MeDH not only can bind methanol with very high affinity, it can also oxidize formaldehyde, indicating that Xox-MeDH transforms methanol directly to formate (146). For methanotrophs that rely on the CBB cycle for carbon fixation, such a phenomenon is of little consequence, as formate can be oxidized further to CO_2 . Methanotrophs that rely on the ribulose monophosphate (RuMP) or serine cycle for carbon assimilation, however, must produce formaldehyde for growth. For example, methanotrophs, such as *M. trichosporium* OB3b, that utilize the serine cycle convert formaldehyde to methylene tetrahydrofolate that is then inserted into the serine cycle. Methylene tetrahydrofolate, however, can also be formed via the condensation of formate with tetrahydrofolate, followed by subsequent reduction to methylene tetrahydrofolate (Fig. 2). In this pathway, formate is first converted to formyl-tetrahydrofolate via the formate tetrahydrofolate ligase (FtfI). Formyl-tetrahydrofolate is then reduced to methylene tetrahydrofolate via a two-step process mediated by methenyltetrahydrofolate cyclohydrolase (FchA) and methylene

tetrahydrofolate dehydrogenase (MtdA). Genes encoding all of these steps (*ftfl*, *fchA*, and *mtdA*), however, were not upregulated when *M. trichosporium* OB3b was grown in the presence of cerium (84), suggesting that either these enzymes are highly active or that formate is not the primary product of methanol oxidation by Xox-MeDH in *M. trichosporium* OB3b. It has been suggested that Xox-MeDH may, depending on the microbe, release formaldehyde rather than formate (146). Probing the metabolome of *M. trichosporium* OB3b and other methanotrophs that rely on either the RuMP or serine pathway and that are solely expressing Xox-MeDH is likely to be very informative in resolving the product(s) of Xox-MeDH *in vivo* for these methanotrophs.

Environmental significance of Xox-methanol dehydrogenase. Recently, it has been shown that not only are genes encoding Xox-MeDH common to many environmental samples (e.g., see references 147–153), they exhibit much greater diversity than Mxa-MeDHs (146). Such findings mean that REE-containing MeDHs might in fact be more environmentally relevant than the “classical” or earlier-characterized Mxa-MeDH. Indeed, methanotrophs have been found to possess only Xox-MeDH and not Mxa-MeDH (Table 1) (154–158). Further, recent findings show that not only is *xoxF* widespread in marine environments (147), it is one of the most abundant transcripts found in marine methylotrophs (159). In addition, dissolved amounts of light REEs substantially decreased in the methane plume associated with the Deepwater Horizon blowout of 2010, and methane consumption correlated with light-REE depletion (160). Collectively, these data strongly imply that Xox-MeDH may be the predominant form of MeDH expressed *in situ*, suggesting that it confers a selective advantage over the Mxa-MeDH for growth on C₁ compounds. Indeed, it has been shown that when expressing Xox-MeDH, the methanotroph *Methylobacter tundripaludum* did not excrete methanol, but it did to a level of 1.24 mM when Mxa-MeDH was expressed, likely due to the poorer affinity for methanol exhibited by the Mxa-MeDH (161). As such, the expression of Xox-MeDH could improve methanotrophic growth yield and/or carbon conversion efficiency by limiting the loss of methanol.

WHAT ARE THE ENVIRONMENTAL ROLES OF THE COPPER AND REE SWITCHES?

Although the copper switch is evident in the laboratory, it is less clear what benefit(s) it might provide for methanotrophic growth *in situ*. One can speculate that when methane is plentiful, methanotrophic biomass will increase, reducing the overall copper-to-biomass ratio, thereby inducing the expression of sMMO, which has a higher turnover rate of methane but weaker affinity than pMMO (27). Conversely, if methane concentrations are low, methanotrophic growth would be expected to be reduced, resulting in diminished overall biomass. Methanotrophs that express the copper switch, however, may continue to predominate by expressing pMMO under these conditions. A question, however, that has been largely ignored in the scientific literature is why do so few known methanotrophs have the copper switch, particularly if one accepts the hypothesis that it provides greater flexibility for these methanotrophs to thrive under varied methane availabilities? This cannot be unequivocally answered at this time, but it may be that the copper switch comes at some fitness cost, preventing methanotrophs exhibiting it from effectively competing with other methanotrophs that only express one form of MMO when methane concentrations are at the extremes (i.e., very low or very high).

For the REE switch, as mentioned earlier, there is clear evidence that the expression of Xox-MeDH can be advantageous to methanotrophs, as it can limit the loss of methanol (161). As such, it would appear that Xox-MeDH expression *in situ* would provide methanotrophs with a competitive advantage, especially as REEs are common, as noted earlier (106). However, in simple two-member communities where a methanotroph (*Methylobacter tundripaludum*) was incubated in the presence of a methylotroph (either *Methylothera mobilis* 13 or *M. mobilis* JW8) with methane provided as the sole carbon source and in the presence of 30 μ M lanthanum (as a representative REE), Mxa-MeDH expression by *M. tundripaludum* increased and Xox-MeDH expression decreased compared to when *Methylobacter tundripaludum* was grown axenically in the

presence of lanthanum (161). It appears that the methylotroph, through the secretion of some soluble compound(s), either limited REE uptake by methanotrophs, or that this compound(s) acted as some signal to override the REE switch. Clearly, in these systems, it is beneficial for the methylotroph to induce the expression of Mxa-MeDH in the methanotrophs to increase methanol secretion and allow cross-feeding to occur. What is less clear is what if any benefit does the methanotroph receive in kind? It has been reported that methane oxidation by a single methanotroph (*Methylobacterium methanica*) increased in artificial mixed communities with increasing heterotroph richness (162), suggesting that the heterotrophs provide some metabolite(s) that promotes methanotrophic growth, e.g., perhaps cobalamin that can stimulate methanotrophic growth (163). It may be that having the flexibility to express either form of MeDH enables methanotrophs to more positively interact with important microbial partners to the benefit of all.

CONCLUSIONS

Methanotrophy continues to offer up surprises, and the recent findings of multiple metal switches in these intriguing microbes provide new opportunities to manipulate these microbes for a variety of opportunities. Despite our expanded knowledge on metal-methanotroph interactions, there is still much we do not know, including the following questions.

What is the complete basis of both the copper switch and REE switch in methanotrophs? Such information could be generated via selective knockouts of suspected regulatory genes identified via transcriptomic analyses. Can we then manipulate these switches in some way to enhance methane valorization? Elucidation of these switches could also be of use in synthetic biology by expanding the “toolbox” available for the construction of robust genetic circuits with unique input parameters.

What genes are required for methanobactin biosynthesis from a precursor polypeptide? A two-pronged approach could be used here. First, methanotrophic mutants could be created where individual genes suspected to be involved in MB biosynthesis are knocked out, followed by characterization of MB intermediates made by these mutants. With such information, the biosynthetic pathway of MB could be reconstructed, and indeed, work has begun using this approach (75). Second, heterologous expression of MB could be attempted where various combinations of genes known or suspected to be involved in MB biosynthesis are inserted in hosts, such as *Escherichia coli*.

How is methylmercury degraded by methanotrophs expressing methanobactin? Stable isotope labeling of methylmercury to follow products of methylmercury degradation would be very useful here, as well as knockouts of gene(s) suspected to be involved. Once the mechanism of methanotrophic-mediated methylmercury degradation is identified, this could then be used as a signature to determine how prevalent such an ability is *in situ*.

How widespread is copper competition *in situ*? How does this impact the emissions of not only methane but also nitrous oxide? Future studies should consider a combination of simple soil microcosm studies where methanotrophs and denitrifiers are cocultured to delineate methanotroph-denitrifier competition for copper, as well as more complex field studies where activities of these microbes are carefully monitored and correlated to net emissions of both methane and nitrous oxide.

How do methanotrophs collect REEs that most commonly are found in extremely insoluble forms? As noted, there are several possibilities, but at this moment, it is still unclear what the mechanism(s) may be. One strategy to begin identifying the mechanism could be to incubate methanotrophs in the presence of different dissolved concentrations of REEs, e.g., REEs in the presence/absence of chelating agents, such as nitrilotriacetic acid, and then compare metal uptake as well as the transcriptome/proteome of these cultures. Doing so may generate clues as to the putative REE uptake system. If this is successful, it may be possible to use this information to enhance the extraction/purification of REEs.

Answering these questions, although likely to be challenging, is also likely to be of great value.

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ADDENDUM IN PROOF

After revising the manuscript, L. Cao, O. Caldararu, A. C. Rosenzweig, and U. Ryde published an article (Angew Chem Int Ed 57:162–166, 2018, <https://doi.org/10.1002/anie.201708977>) concluding, using quantum refinement studies, that the proposed di-copper active site of pMMO (36) does not exist in the crystal structure of this enzyme. Rather, this site is best modeled as a mono-copper site. As such, we believe it important to reiterate that much more work is required to understand the mechanism of methane oxidation by pMMO.

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