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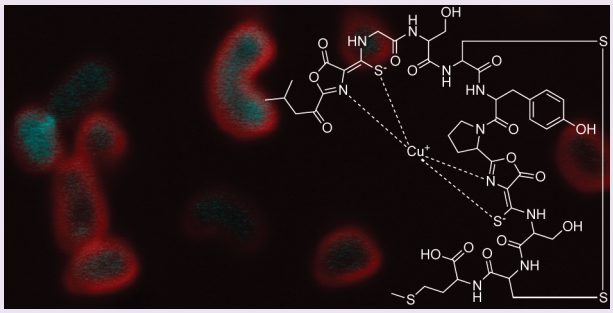
Chemistry and Biology of the Copper Chelator Methanobactin

Grace E. Kenney and Amy C. Rosenzweig*

Departments of Molecular Biosciences and of Chemistry, Northwestern University, Evanston, Illinois 60208, United States

ABSTRACT: Methanotrophic bacteria, organisms that oxidize methane, produce a small copper chelating molecule called methanobactin (Mb). Mb binds Cu(I) with high affinity and is hypothesized to mediate copper acquisition from the environment. Recent advances in Mb characterization include revision of the chemical structure of Mb from *Methylosinus trichosporium* OB3b and further investigation of its biophysical properties. In addition, Mb production by several other methanotroph strains has been investigated, and preliminary characterization suggests diversity in chemical composition. Initial clues into Mb biosynthesis have been obtained by identification of a putative precursor gene in the *M. trichosporium* OB3b genome. Finally, direct uptake of intact Mb into the cytoplasm of *M. trichosporium* OB3b cells has been demonstrated, and studies of the transport mechanism have been initiated. Taken together, these advances represent significant progress and set the stage for exciting new research directions.



Copper plays an important role in many bacteria, serving as a cofactor in essential enzymes such as cytochrome oxidase, NADH dehydrogenase, and superoxide dismutase.[1] An array of proteins, including metallochaperones, metalloregulatory proteins, P_{1B}-type transport ATPases, and proton-substrate antiporters,[2−6] ensures that copper is provided to these enzymes but does not accumulate to toxic levels.[7] Most of these homeostatic proteins are involved in removal of excess copper, and it remains largely unclear how bacteria import necessary copper.[3,4]

Copper acquisition is particularly important for methanotrophs, Gram-negative bacteria that utilize methane as their sole carbon source.[8] The first step in methanotroph metabolism is the oxidation of methane to methanol by the copper-dependent enzyme particulate methane monooxygenase (pMMO).[8−12] pMMO is membrane-bound[13,14] and accounts for up to 20% of the total cellular protein in methanotrophs.[15] Not only is copper catalytically important for pMMO, but in some methanotroph strains, copper also controls expression of an alternate iron-dependent[16,17] methane oxidation system, soluble methane monooxygenase (sMMO). In these methanotrophs, copper represses transcription of the sMMO genes and leads to formation of intracytoplasmic membranes that house pMMO.[11,13,18] This "copper switch" also affects expression of various other proteins.[8] Although there has been some progress characterizing MmoR and MmoG, two proteins involved in copper-dependent transcriptional regulation of sMMO,[19] the molecular mechanism of the copper switch and how it affects pMMO regulation, Mb biosynthesis, and intracellular membrane formation remain unknown.

To meet their high requirement for copper, methanotrophs produce and secrete small copper chelating molecules. The existence of these copper binding compounds, originally called CBCs, was first postulated from studies of *Methylosinus trichosporium* OB3b mutant strains that do not switch from sMMO to pMMO expression under copper replete conditions.[20,21] Small molecules (382−1218 Da) that bind copper with high affinity were subsequently isolated from the spent media of several strains of methanotrophs under copper starvation conditions.[22−24] A pure sample of one such CBC was obtained from *M. trichosporium* OB3b[25,26] and its crystal structure determined.[27] This initial structure revealed a 1217 Da molecule with the sequence *N*-2-isopropyl ester-(4-thionyl-5-hydroxyimidazolate)-Gly[1]-Ser[2]-Cys[3]-Tyr[4]-pyrrolidine-(4-hydroxy-5-thionylimidazolate)-Ser[5]-Cys[6]-Met[7] (Figure 1a). A single copper ion, determined to be Cu(I) from X-ray photoelectron (XPS),[27] X-ray absorption (XAS),[28] and electron paramagnetic resonance (EPR)[28,29] spectroscopic data, is coordinated by two nitrogen and two sulfur atoms.

The compound was named methanobactin (Mb) and is sometimes referred to as a "chalkophore" because its peptidic nature resembles many iron siderophores (*chalko-* is derived from the Greek word for copper; *sidero-* is from the Greek word for iron).[27] The primary function of Mb is proposed to be copper uptake, particularly from insoluble mineral sources.[30,31] It is widely believed that Mb is secreted in the apo form (apo-Mb) to obtain copper from the environment and then internalized in its copper bound form (Cu-Mb). Many aspects of this model have not been tested in detail, however. Ancillary functions of Mb have also been proposed, including oxidative stress defense[32] and enhancing pMMO activity,[29] although strong evidence for the latter has not been obtained.[33]

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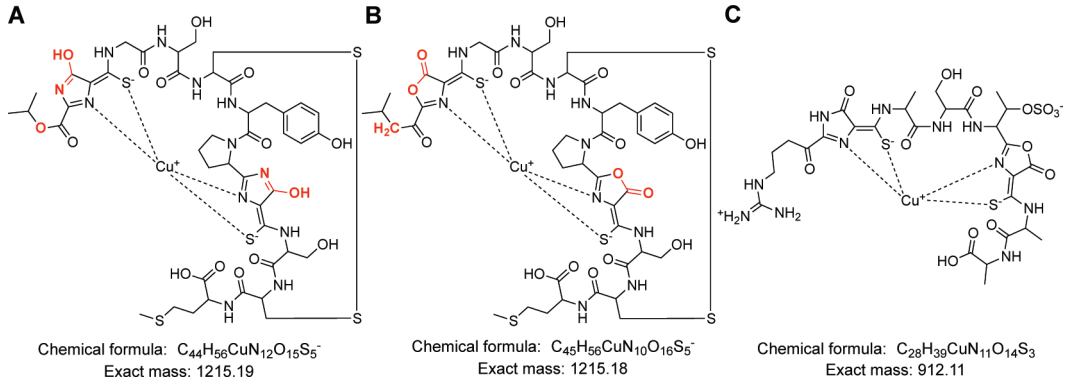

Figure 1. Methanobactin structures. (a) Initial structure of *M. trichosporium* OB3b Cu-Mb determined by X-ray crystallography. (b) Revised solution structure of *M. trichosporium* OB3b Cu-Mb. Differences between the two structures are highlighted in red. (c) Proposed structure of *Methylocystis* strain SB2 Cu-Mb.

Since the last comprehensive review of Mb in 2008,[34] the molecular structure of *M. trichosporium* OB3b Mb has been revised and its biophysical properties have been investigated further. In addition, significant progress has been made toward addressing three key questions surrounding Mb structure and function. First, the question of whether other methanotroph strains produce Mb, and if so, whether it has the same structure and chemical composition, has been pursued vigorously. Second, some initial clues into Mb biosynthesis have been obtained. Third, the proposed copper acquisition function of Mb has been investigated experimentally, providing direct evidence for copper uptake via Mb as well as some insight into the nature of the uptake machinery. These recent studies, summarized here, represent major advances and suggest new research directions for the field.

ISOLATION AND CHARACTERIZATION OF MB FROM *M. TRICHOSPORIUM* OB3B

Several detailed protocols are now available for isolation of *M. trichosporium* OB3b Mb.[35,36] In general, cells are grown either with no copper or with low levels of copper in the media and the spent media is harvested by centrifugation. The spent media is then concentrated using either reversed-phase tC18 Sep-Pak cartridges or, for a larger scale, a Diaion HP20 column. Further purification is accomplished by reversed-phase HPLC. Typically, Cu(II) is added immediately after isolation of the spent media to stabilize Mb, although the less stable apo-Mb can also be isolated.[35−37] The integrity of purified Cu-Mb can be assessed by optical spectroscopy and mass spectrometry. According to mass spectrometry analysis, the preparations typically contain some fraction of a variant that is missing the C-terminal methionine residue, Met^7. This material, which structurally, spectroscopically, and functionally resembles intact Cu-Mb, is present in variable amounts, and its presence has not been correlated with specific growth conditions.[35,38]

Since 2008, two new structures of *M. trichosporium* OB3b Cu-Mb have been reported. The solution structure revealed two important differences from the X-ray structure (Figure 1b).[39] First, the NMR data are consistent with an N-terminal isobutyl group rather than an isopropyl group. Second, analysis of the ^{15}N spectrum indicates that the originally assigned hydroxyimidazolate rings are instead oxazolone rings. This revised structure is consistent with mass spectrometric data. The sequence of *M. trichosporium* OB3b Mb is therefore now believed to be 1-(*N*-[mercapto-{5-oxo-2-(3-methylbutanoyl)-oxazol-(*Z*)-4-ylidene}methyl]-Gly^1-Ser^2-Cys^3-Tyr^4)-pyrrolidin-2-yl-(mercapto-[5-oxo-oxazol-(*Z*)-4-ylidene]methyl)-Ser^5-Cys^6-Met^7 (Figure 1b). In addition, the crystal structures of intact Cu-Mb and Cu-Mb lacking the C-terminal methionine were determined to 0.92 and 1.00 Å resolutions, respectively.[38] These structures are very similar to the original 1.15 Å resolution structure[27] and are consistent with the differences in chemical composition indicated by the NMR data. Loss of the C-terminal methionine does not affect the structure significantly, and the new C-terminal residue, Cys^6, remains in a disulfide bond with Cys^3.[38]

Several biophysical properties of *M. trichosporium* OB3b Cu-Mb have been further investigated as well. Molar extinction coefficients for apo-Mb (340 nm, 22.9 mM^{-1} cm^{-1}; 394 nm, 22.1 mM^{-1} cm^{-1}) and Cu-Mb (290 nm, 19.5 mM^{-1} cm^{-1}) have been measured and reported.[38] The features at 340 and 394 nm are assigned to the oxazolone rings.[39] The oxidation state of the two cysteine residues was also explored. Neither apo-Mb nor Cu-Mb reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), indicating that the disulfide bond is present in both forms,[38] consistent with earlier studies.[22,26] Thus, reduction of Cu(II) to Cu(I) by Mb is not coupled to disulfide bond formation, as suggested previously.[28] The Cu(I) binding affinity of all forms of Mb, determined by competition experiments with bathocuproine disulfonate (BCS), is $(6–7) \times 10^{20}$ M^{-1}.[38] This value is several orders of magnitude higher than the affinity of the copper chaperones Atx1 and Atox1 for Cu(I)[40] and raises the question of how Cu-Mb releases its Cu(I) ions. Finally, reduction potentials at pH 7.5 of 640 and 590 mV versus NHE were measured for Cu-Mb and Cu-Mb lacking the C-terminal methionine, respectively.[38]

DIVERSITY OF MB

The production of Mb by methanotrophs other than *M. trichosporium* OB3b has been a major research focus over the past three years. One approach has been the development of an assay to detect Mb production in growing methanotroph cultures. The chrome azurol S (CAS) assay, often used to detect iron siderophores,[41] was adapted to screen for Mb.[42,43] In this protocol, the disappearance of a blue Cu-CAS complex is monitored either in solution by optical spectroscopy or on agar plates by eye. If Mb is produced, it removes copper from CAS, leading to a color change from blue to orange. Experiments must be performed in parallel using Fe-CAS to avoid false positive results from Cu-siderophore complexes.

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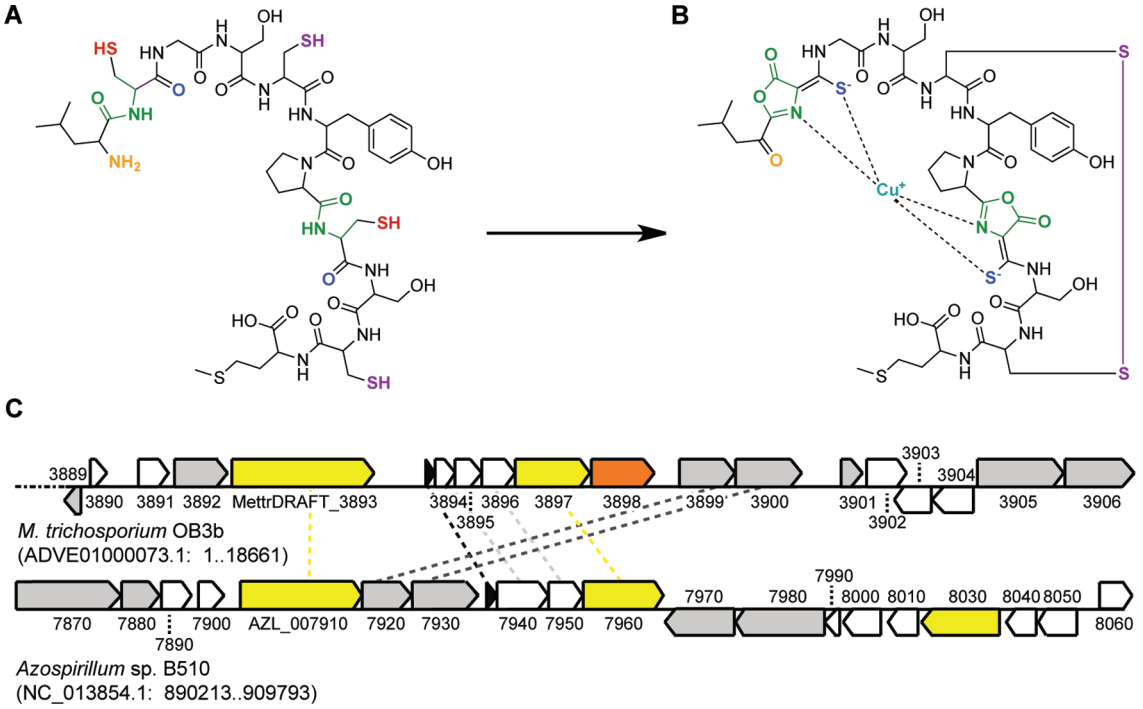

Figure 2. Possible pathway for biosynthesis of *M. trichosporium* OB3b Mb. (A) Putative precursor peptide without leader sequence. (B) *M. trichosporium* OB3b Cu-Mb. Necessary modifications would include replacement of the N-terminal amine group with a carbonyl group (orange), replacement of two cysteines with serines (red) prior to cyclization and formation of the oxazolone rings (green), and replacement of the oxygens in the carbonyl groups adjacent to the two initial cysteines with sulfur to form thioamide groups (blue). A disulfide bond must then be formed (purple) and the resultant apo-Mb loaded with copper (cyan). (C) Genomic neighborhood of the putative precursor gene in *M. trichosporium* OB3b and *Azospirillum* sp. B510 genomes. The *M. trichosporium* OB3b genome has not been fully assembled, and the location of the precursor peptide-containing 18.66 kb contig in relation to the other 172 contigs is not known. The *Azospirillum* sp. B510 genome is complete; an 18.66 kb region around the precursor peptide is displayed for purposes of comparison. Full-length precursor peptides are shown in black, genes with no predicted annotated function are shown in white, genes with predicted annotated functions but unclear relevance to Mb are shown in gray, transporter genes are shown in yellow, and an aminotransferase gene is shown in orange. Potential homologues in the two genomes are connected by dashed lines.

According to this assay, *Methylococcus capsulatus* (Bath) and *Methylomicrobium album* BG8 produce Mb, but *Methylocystis parvus* OBBP does not.[42] Neither *M. album* BG8 nor *M. parvus* OBBP have an sMMO system. Interestingly, siderophores are also secreted by *Methylocystis* species strain M, *M. trichosporium* OB3b and *M. album* BG8, but not by *M. capsulatus* (Bath) and *M. parvus* OBBP.[42,44]

Consistent with the results of the Cu-CAS assay, candidate Mb molecules have been isolated from *M. capsulatus* (Bath) and *M. album* BG8[33,45] with molecular masses of 1056.48 and 939.33 Da, respectively.[45] These putative Mb molecules are less abundant than *M. trichosporium* OB3b Mb, and the amount present is apparently not correlated with initial copper concentrations in the media. The optical spectra of these Mb samples also differ significantly from that of *M. trichosporium* OB3b Mb. *M. trichosporium* OB3b Cu-Mb exhibits distinct features at 340 and 394 nm, and *M. trichosporium* OB3b apo-Mb exhibits features in the 250−300 nm range.[25,26,38] By contrast, the apo forms of Mb from the other two organisms display weak features at 260−270 and 400 nm, and an increase at 400 nm is observed upon copper addition. The fluorescence properties are similar to those of *M. trichosporium* OB3b Mb, although less quenching is observed upon copper addition. EPR analysis indicates that ∼80% of the copper bound to these Mb molecules is Cu(I). Finally, these compounds bind copper with moderate affinities of 10^5−10^6 M^{-1}, as determined by isothermal titration calorimetry.[45] These values are not consistent with the high affinity of CAS for copper or the bindng constant of (6−7) × 10^{20} M^{-1} reported for *M. trichosporium* OB3b Mb,[38] but comparing copper binding constants measured by different methods is generally fraught with technical difficulties.[40] Given the lower abundance of these molecules, the uncertainty of the link between their production and copper availability, and the lack of structural information, more data will be required to identify them definitively as novel forms of Mb. It should also be noted that methanotrophs secrete other molecules in this molecular mass range, including flavins and siderophores.[42,44]

The Mb from *Methylocystis* strain SB2, a recently isolated methanotroph that produces only pMMO and does not switch to sMMO upon copper depletion,[46] has been characterized in more detail.[47] The mass of *Methylocystis* strain SB2 Mb is 851.20 Da for the apo form and 912.11 Da for a sample with a single bound copper ion. The oxidation state of this copper ion was assigned as Cu(I) on the basis of XPS data. The optical spectrum reveals peaks at 338 and 387 nm, which are similar to the 340 and 394 nm features observed for *M. trichosporium* OB3b Mb. The chemical composition of *Methylocystis* strain SB2 Mb has been investigated by NMR spectroscopy, and a partial sequence has been proposed. This sequence includes four intact amino acid residues, one oxazolone ring, one imidazolone ring, and modified arginine and threonine residues (Figure 1c). This structure has a calculated mass of 771.24 Da, which can be reconciled with the measured mass if a sulfonate group is present. It is suggested that this sulfonate group is appended to the threonine-like side chain (Figure 1c).[47] Thus,

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Methylocystis strain SB2 Mb shares some structural features with *M. trichosporium* OB3b Mb, including the apparent coordination of Cu(I) by two five-membered heterocyclic rings and neighboring thioamide groups, but is smaller with a different amino acid composition.

BIOSYNTHESIS OF MB

The availability of the *M. trichosporium* OB3b genome in 2010[48] combined with the revised *M. trichosporium* OB3b Mb structure[39] has provided new insight into Mb biosynthesis. One proposed route for Mb biosynthesis involves a nonribosomal peptide synthetase (NRPS) system.[25,34] NRPS systems mediate siderophore biosynthesis[49] and are present in both the *M. trichosporium* OB3b[48] and, to a much lesser extent, *M. capsulatus* (Bath)[50] genomes. A second possibility is that Mb is generated by modification of a ribosomally synthesized peptide.[39] Preliminary evidence for this proposed route derives from identification in the *M. trichosporium* OB3b genome of a candidate precursor gene that encodes the peptide MTVKIAQKKVLPVIGRAAA**LCGSCYPCSCM**[47] (residues shown in bold are part of Mb; other residues may be a "leader sequence" as present in other natural products derived via posttranslational modification[51]) (Figure 2a). This peptide resembles a previously proposed starting peptide, LSGSCYPSSCM,[39] with the substitution of cysteines for two of the serines. In that proposed mechanism, a set of posttranslational modification steps were suggested by analogy to biosynthesis of thiazolyl antibiotics such as microcin B17.[39,52] A similar mechanism can be envisioned for the peptide precursor LCGSCYPCSCM (Figure 2b), with the additional requirement that the two cysteines be converted to serines to allow formation of oxazolone rather than thiazolone rings.[47] This conversion could be followed by a series of sulfuration, cyclization, and transamination steps to form the final compound (Figure 2b). There are precedents in other biosynthesis systems for cyclization of serines[53] and for transamination reactions.[54] The Mb from *Methylocystis* strain SB2 could be synthesized by a related series of modifications.[47]

The putative Mb precursor gene was not previously identified as a coding region and is found between genes MettrDRAFT_3893 and MettrDRAFT_3894 on Contig 00014 (NCBI reference sequence ADVE01000073.1) of the *M. trichosporium* OB3b draft genome (Figure 2c).[47] Nearby genes encode a mutator type transposase (MettrDRAFT_3889) on the reverse strand and a neighboring hypothetical protein (MettrDRAFT_3890) on the forward strand, a sigma factor and FecR pair (MettrDRAFT_3891 and MettrDRAFT_3892), a TonB-dependent transporter (MettrDRAFT_3893), several unidentified and hypothetical proteins (MettrDRAFT_3894−3896), a member of the multidrug and toxic compound extrusion (MATE) family (MettrDRAFT_3897), an aminotransferase (MettrDRAFT_3898), a diheme enzyme (MettrDRAFT_3900) predicted to be a cytochrome *c* peroxidase and related to CorB from *M. album* BG8,[55] and another gene product probably associated with the diheme enzyme (MettrDRAFT_3899) (Table 1). A sigma factor (MettrDRAFT_3901) and the unknown protein that follows it (MettrDRAFT_3902) are the last consecutive coding regions found on the forward strand. There are four other coding sequences on the contig, two unidentified putative proteins (MettrDRAFT_3903 and MettrDRAFT_3904) on the reverse strand followed by two genes related to type I restriction-modification systems (MettrDRAFT_3905 and MettrDRAFT_3906) (Table 1). It is not clear whether any of these proteins play a role in Mb biosynthesis or handling.

Table 1. Annotated Genes Near the Putative Precursor Peptides of *M. trichosporium* OB3b and *Azospirillum* sp. B510

Gene	Annotation
MettrDRAFT_3889	Transposase, mutator type
MettrDRAFT_3891	RNA polymerase sigma 24 subunit, ECF subfamily
MettrDRAFT_3892	FecR iron sensor protein
MettrDRAFT_3893	TonB-dependent transporter
MettrDRAFT_3897	Multidrug antimicrobial extrusion (MATE) protein
MettrDRAFT_3898	Aminotransferase class I and II
MettrDRAFT_3899	Similar to CorA (*M. album* BG8); part of two-gene unit with diheme enzyme
MettrDRAFT_3900	Diheme cytochrome *c* peroxidase, similar to CorB (*M. album* BG8)
MettrDRAFT_3901	RNA polymerase sigma 24 subunit, ECF subfamily
MettrDRAFT_3905	N-6 DNA methylase (type I restriction-modification system, M subunit)
MettrDRAFT_3906	Type I restriction-modification system, DNA specificity domain (S subunit)
AZL_007870	Putative uncharacterized protein (contains domains similar to CopC and CopD)
AZL_007880	SCO2 protein
AZL_007910	TonB-dependent transporter
AZL_007920	Similar to CorA (*M. album* BG8); part of two-gene unit with diheme enzyme
AZL_007930	Diheme cytochrome *c* peroxidase, similar to CorB (*M. album* BG8)
AZL_007960	Multidrug antimicrobial extrusion (MATE) protein
AZL_007970	CydB (cytochrome bd-I oxidase subunit II)
AZL_007980	CydA (cytochrome bd-I oxidase subunit I)
AZL_008030	TonB-dependent transporter

A similar precursor peptide is found in only one other genome, that of the rice endophyte *Azospirillum* sp. B510.[47,56] The significance of the absence of homologues in other sequenced methanotroph genomes is unclear but may reflect the structural diversity of Mb molecules or the ability of methanotrophs to scavenge Mbs from other species. In the case of the *Azospirillum* sp. B510 genome, a 31-residue coding region encodes the peptide MTIKIAKKQTLSVAGRAGACCGSCCAPVGVN.[47,56] Several of the neighboring genes observed in the *M. trichosporium* OB3b genome are also conserved in *Azospirillum* sp. B510,[47] including the TonB-dependent transporter (AZL_007910), the MATE family member (AZL_007960), the diheme enzyme and its neighbor (AZL_007930 and AZL_007920), and the unknown proteins MettrDRAFT_3894−3896 (AZL_007940, which has separate regions homologous to MettrDRAFT_3894 and MettrDRAFT_3895, and AZL_007950) (Figure 2c, Table 1). The significance of this similarity remains unclear, however. It is potentially relevant that *Azospirillum* sp. B510 produces siderophores.[56]

COPPER UPTAKE FUNCTION

The proposed copper uptake function of Mb derives from early observations linking the presence of Mb in the spent media with copper limitation during growth.[22−24] In support of a role in copper uptake, Mb can promote the switch from sMMO expression to pMMO expression, as demonstrated by monitoring protein levels, by monitoring transcript levels of *pmoA* (encodes pMMO pmoA subunit) and *mmoX* (encodes sMMO α subunit), or by detecting sMMO activity.[30,36,38]

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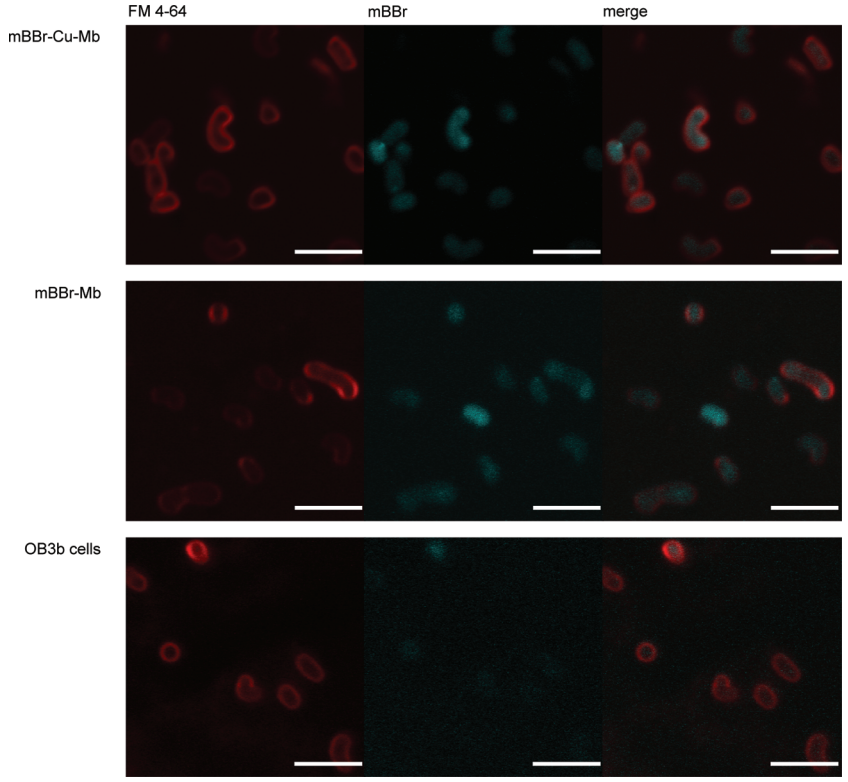

Figure 3. Imaging Mb uptake by confocal microscopy. Fluorescence images were taken at 405 nm (cyan, mBBr-Mb) and 543 nm (red, the lipophilic membrane dye FM 4-64). The merged images show the distribution of both signals. The scale bar corresponds to 4 μm. *M. trichosporium* OB3b cells incubated with mBBr-Cu-Mb and FM 4-64 (top) or mBBr-Mb and FM 4-64 (middle) exhibit primarily cytoplasmic localization of the compound. The background fluorescence of *M. trichosporium* OB3b cells, which presumably includes the intrinsic fluorescence of internally produced Mb (bottom, also in the presence of FM 4-64) is also detectable but is of a much lower magnitude.

Whereas pMMO has a limited substrate specificity, sMMO oxidizes a number of larger substrates,[57] including naphthalene, which is amenable to a colorimetric assay.[58] A copper-mediated switch from sMMO to pMMO can be observed in *M. trichosporium* OB3b upon addition of copper as either $CuCl_2$ or Cu-Mb, but only Cu-Mb stimulates the switch in the presence of Cu-doped Fe oxide or Cu-doped borosilicate glass,[30] and Cu-Mb can actually increase the dissolution rate of Cu-substituted glass.[31] These findings provide insight into Mb function in nature and have been extended to correlate copper content of glass with methane oxidation rates of *M. trichosporium* OB3b.[59] In these experiments, methane depletion was measured in the presence of manufactured borosilicate glasses with 0−800 ppm Cu, with maximum rates observed at 80 and 200 ppm, and the lowest rates at 0 and 800 ppm. The methane oxidation rates are comparable to those measured using soluble copper, suggesting that insoluble copper can support methanotroph growth in nature. The current direction for this work is to assess the effect of Mb on growth using copper mineral sources other than Cu-doped silicates, including malachite, tenorite, cuprite, and chalcocite.[60]

Although these data strongly support the copper uptake function, direct evidence for uptake of the Mb molecule has been lacking, and the specific mechanisms by which copper from Cu-Mb is internalized have not been established. These issues have been addressed recently by tracking copper and Mb uptake by isotopic and fluorescent labeling.[61] An increase in intracellular copper content of *M. trichosporium* OB3b cells treated with ^{65}Cu-Mb was correlated with an altered ^{63}Cu/^{65}Cu ratio, indicating that Cu bound to Mb is internalized. The high affinity of Mb for copper suggests that intact Cu-Mb is taken up, and the labeling of Cu-Mb with the fluorescent probe monobromobimane (mBBr-Cu-Mb) permitted verification of mBBr-Cu-Mb uptake into the cytoplasm by confocal microscopy (Figure 3). Interestingly, the apo form of labeled Mb (mBBr-Mb) is also taken up by *M. trichosporium* OB3b cells (Figure 3). Other methanotroph strains, including *Methylocystis* sp. strain M, *M. capsulatus* (Bath), and *Methylomicrobium alcaliphilum* 20Z also internalize Cu-Mb from *M. trichosporium* OB3b, but *E. coli* does not, suggesting that the phenomenon is specific to methanotrophs.[61]

Initial insight into the mechanism of Cu-Mb uptake has also been obtained. In Gram-negative bacteria, nutrients are transported across the outer membrane either by passive diffusion using porins or by active transport using TonB-dependent transporters (TBDTs).[62] TBDTs interact with the inner membrane TonB-ExbB-ExbD protein complex and transduce energy from the inner membrane proton motive force to translocate substrates across the outer membrane. These systems are involved in siderophore uptake[63] and have been suggested as potential Cu-Mb transporters.[34] Porins and TBDTs can be inhibited selectively using spermine and uncouplers such as cyanide *m*-chlorophenyl hydrazone (CCCP) or methylamine, respectively. Treatment of *M. trichosporium* OB3b cells with spermine inhibits uptake of unchelated copper (provided as $CuSO_4$), but not Cu-Mb, and treatment with CCCP or methylamine inhibits uptake of Cu-Mb, but not unchelated copper.[61] Therefore, Cu-Mb is not transported by porins, but in an energy-dependent process that might involve TBDTs (Figure 4). The existence of specific

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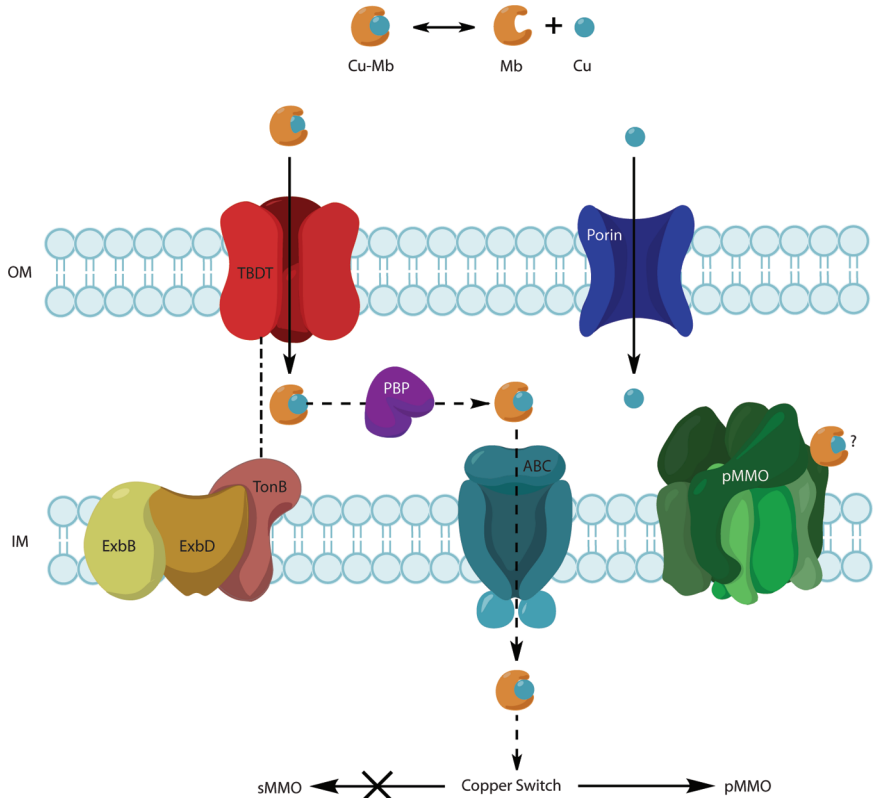

Figure 4. Hypothesized uptake mechanisms for methanobactin and copper. Copper (Cu) is transported across the outer membrane (OM) by a passive transport process likely involving porins, whereas Cu-Mb is transported via an active process potentially mediated by TonB-dependent transporters (TBDTs) and their interactions with the TonB-ExbB-ExbD complex. The mechanism of translocation across the inner membrane (IM) is unknown but, by analogy to siderophores, could involve an ATP-binding cassette (ABC) transporter and a periplasmic binding protein (PBP). It remains unclear whether intact Cu-Mb interacts directly with pMMO or with proteins involved in the "copper switch".

outer membrane receptors for Mb is supported by the observation that apo-Mb can compete with Cu-Mb for copper uptake.[61]

Finally, Mb may have practical applications beyond its copper uptake function in methanotrophs. For example, *M. trichosporium* OB3b Mb was recently tested as a therapeutic in a rat model of Wilson disease, a human genetic disorder characterized by toxic copper overload in the liver due to reduced biliary excretion.[64] In this study, Mb was demonstrated to remove copper from metallothionein and to promote excretion of copper in bile.[65] Thus, Mb may represent a new route to treatments for Wilson disease. Such efforts may be enhanced by development of synthetic Mb molecules.[66]

SUMMARY

The structure and biophysical properties of *M. trichosporium* OB3b Mb are well understood,[38,39] and internalization of Mb by *M. trichosporium* OB3b and other methanotrophs is now established. Uptake occurs through an active transport process, perhaps using TBDTs.[61] Identifying which, if any, of the approximately 45 TBDTs in the *M. trichosporium* OB3b genome is involved represents a significant future challenge. Moreover, transport of Mb across the inner membrane has not been explored at all, but by analogy to siderophores could conceivably involve ABC transporters and periplasmic binding proteins (Figure 4). How copper is released from Cu-Mb and whether Cu-Mb directly provides copper to the pMMO active site or directly triggers the copper switch also remain unclear. Methanotrophs besides *M. trichosporium* OB3b produce Mb-like molecules that can chelate copper,[42] and in the case of *Methylocystis* strain SB2, preliminary characterization suggests a chemical composition that recapitulates some, but not all, features of *M. trichosporium* OB3b Cu-Mb.[47] Further detailed structural and chemical analyses will be critical to determining the prevalence and diversity of Mb in methanotrophs. A first clue into Mb biosynthesis has been obtained with the identification of a potential precursor peptide in the *M. trichosporium* OB3b genome.[47] A major goal for the future is to determine whether this peptide is indeed involved and if so, to unravel the complete biosynthetic mechanism. Also completely unexplored is the means by which methanotrophs secrete Mb. Addressing these main issues, identification of the transport machinery, structural characterization of new Mb molecules, and elucidating the mechanism of Mb biosynthesis, will be the central focus of Mb research in the next few years.

AUTHOR INFORMATION

Corresponding Author

* E-mail: amyr@northwestern.edu.

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ABBREVIATIONS

apo-Mb: metal free form of methanobactin; CAS: chrome azurol S; CBC: copper binding compound; Cu-Mb: copper bound form of methanobactin; mBBr: monobromobimane; Mb: methanobactin; mBBr-Cu-Mb: Cu-Mb labeled with monobromobimane; pMMO: particulate methane monooxygenase; sMMO: soluble methane monooxygenase; TBDT: TonB-dependent transporter

KEYWORDS

Methanotroph: a type of bacteria that lives on methane as its sole source of carbon and energy; Methane monooxygenase: a metalloenzyme that oxidizes methane to methanol in methanotrophs; Particulate methane monooxygenase: integral membrane methane monooxygenase that contains copper; Soluble methane monooxygenase: cytoplasmic methane monooxygenase that contains a diiron active site; Methanobactin: a small high affinity copper chelating molecule isolated from methanotrophic bacteria; Chalkophore: high affinity copper binding molecule analogous to an iron siderophore; TonB-dependent transporter: outer membrane protein in Gram-negative bacteria that facilitates uptake of nutrients by transducing energy from the inner membrane proton motive force; Wilson disease: a genetic disorder of copper metabolism characterized by toxic copper accumulation in the liver

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