Two TonB-Dependent Transporters in *Methylosinus trichosporium* OB3b Are Responsible for Uptake of Different Forms of Methanobactin and Are Involved in the Canonical "Copper Switch"

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ABSTRACT Copper is an important component of methanotrophic physiology, as it controls the expression and activity of alternative forms of methane monooxygenase (MMO). To collect copper, some methanotrophs secrete a chalkophore- or copper-binding compound called methanobactin (MB). MB is a ribosomally synthesized post-translationally modified polypeptide (RiPP) that, after binding copper, is collected by MbnT, a TonB-dependent transporter (TBDT). Structurally different forms of MB have been characterized, and here, we show that different forms of MB are collected by specific TBDTs. Further, we report that in the model methanotroph, *Methylosinus trichosporium* OB3b, expression of the TBDT required for uptake of a different MB made by *Methylocystis* sp. strain SB2 (MB-SB2) is induced in the presence of MB-SB2, suggesting that methanotrophs have developed specific machinery and regulatory systems to actively take up MB from other methanotrophs for copper collection. Moreover, the canonical "copper switch" in *M. trichosporium* OB3b that controls expression of alternative MMOs is apparent if one of the two TBDTs required for MB-OB3b and MB-SB2 uptake is knocked out, but is disrupted if both TBDTs are knocked out. These data indicate that MB uptake, including the uptake of exogenous MB, plays an important role in the copper switch in *M. trichosporium* OB3b and, thus, overall activity. Based on these data, we propose a revised model for the copper switch in this methanotroph that involves MB uptake.

IMPORTANCE In this study, we demonstrate that different TBDTs in the model methanotroph *Methylosinus trichosporium* OB3b are responsible for uptake of either endogenous MB or exogenous MB. Interestingly, the presence of exogenous MB induces expression of its specific TBDT in *M. trichosporium* OB3b, suggesting that this methanotroph is able to actively take up MB produced by others. This work contributes to our understanding of how microbes collect and compete for copper and also helps inform how such uptake coordinates the expression of different forms of methane monooxygenase. Such studies are likely to be very important to develop a better understanding of methanotrophic interactions via synthesis and secretion of secondary metabolites such as methanobactin and thus provide additional means whereby these microbes can be manipulated for a variety of environmental and industrial purposes.

KEYWORDS methanotrophy, methanobactin, copper, TonB-dependent transporter, microbial interactions, chalkophore

Methane is an important greenhouse gas with a global warming potential 28 to 34 times higher than that of carbon dioxide (1). Approximately one-third of all methane emissions are derived from natural processes, i.e., methanogenesis in pristine environments,

with the rest attributed to human activities, e.g., extraction of fossil fuels, livestock farming, manure management and application, rice cultivation, and solid waste and wastewater treatment (2, 3). Annual global methane emissions have increased by over 25% in the past 40 years, largely due to human activities, making it the second most important greenhouse gas after carbon dioxide (3).

Methanotrophs, a group of microbes that use methane as their sole carbon and energy source, play an important role in eliminating methane emissions and in the global carbon cycle. Methanotrophs are phylogenetically and physiologically quite diverse, i.e., both bacteria and archaea have been found to consume methane both aerobically and anaerobically (4–10). Perhaps the best studied are the aerobic bacterial methanotrophs, i.e., members of the *Proteobacteria* (classes *Gamma-* and *Alphaproteobacteria)* and *Verrucomicrobia* phyla (11–15). These bacteria are widely distributed in diverse terrestrial, aquatic, and marine environments and play a major role in eliminating methane emissions from both natural (e.g., forest soils) as well as engineered environments (e.g., landfills) (4, 16).

The first step of methane metabolism by aerobic methanotrophs is oxidation of methane to methanol. Two forms of methane monooxygenases perform this transformation, the membrane-bound or particulate methane monooxygenase (pMMO, encoded by *pmoCAB* or *pmo* operon) and the cytoplasmic or soluble methane monooxygenase (sMMO, encoded by *mmoXYBZDC* or *mmo* operon). Interestingly, pMMO has a much higher affinity for methane than sMMO, but turns it over much more slowly (17). It may be that methanotrophs that can express both pMMO and sMMO have an advantage for growth *in situ* versus those methanotrophs that can only express one of the two forms (4). Interestingly, the expression and activity of the two forms of methane monooxygenase is not dependent on methane availability but, rather, copper, aka the "copper switch" (18). Under copper-limited conditions, some methanotrophs express sMMO, but as the copper/biomass ratio increases, sMMO expression is repressed, while pMMO expression increases with increasing copper (19–21). Such response is due to the fact that sMMO is a soluble di-iron monooxygenase, while pMMO has multiple metal centers; the exact nature of these is still a matter of debate, with several models proposed, although all agree that copper plays a critical role (22–26).

Some methanotrophs, including species that exhibit the copper switch (i.e., can express both sMMO and pMMO) and species that do not (i.e., only express pMMO), have been found to secrete a copper-binding compound or chalkophore termed methanobactin (MB) for copper uptake. MBs have a high affinity for copper ions and can also extract copper bound to other organic molecules as well as minerals (27–31). MBs are small (<1,300 Da) ribosomally synthesized posttranslationally modified polypeptides (RiPPs) that are characterized by two heterocyclic rings that bind copper via an N_2S_2 ligand set (4, 28, 32–35). MB can be divided into two general groups, groups I and II. Group I MBs have one oxazolone ring and either an oxazolone or pyrazinedione group as the second ring and form a dicyclic pyramidal-like shape after binding copper, e.g., MB from *Methylosinus trichosporium* OB3b (MB-OB3b) (32, 33, 36). Group II MBs have one oxazolone group with the second ring either an imidazolone or pyrazinedione ring, and they adopt a hairpin-like shape after binding copper, e.g., MB from *Methylocystis* sp. strain SB2 (MB-SB2) (27, 32). Recent phylogenetic analyses suggest that the two general groups can be further divided into several subgroups, i.e., groups IA, IB, IIA, IIB, and IIC (37).

The gene encoding the polypeptide precursor of MB, *mbnA*, has been identified in a number of methanotrophs (27, 32, 34, 38). *mbnA* is part of the *mbn* gene cluster that includes genes involved in maturation of the MB polypeptide precursor and transport (Fig. 1a) (34, 37, 39, 40). Like sMMO and pMMO, expression of *mbn* genes is copper regulated, i.e., expression is greatest when copper is not provided and decreases when copper is added, i.e., expression of these genes is coregulated with sMMO and reciprocally regulated with pMMO (34, 38, 41–43). Based on various transcriptional studies, a variety of regulatory models coordinating expression of MMOs and MB have been postulated (32, 34, 42, 44).
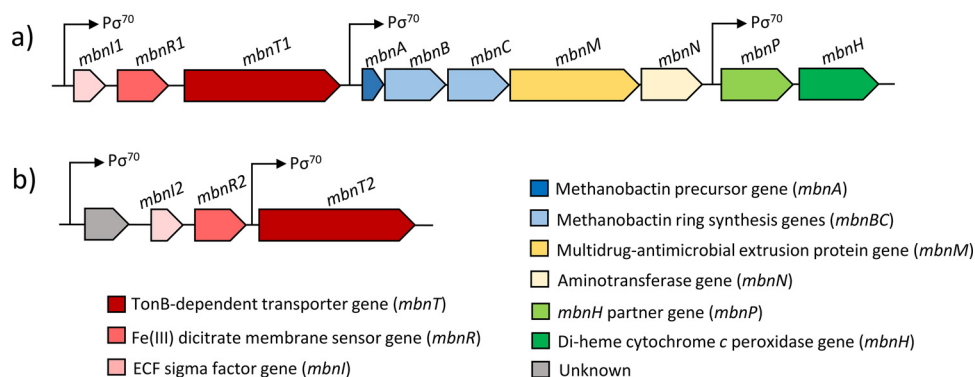
FIG 1 Methanobactin (a) and *mbnT2* gene (b) clusters of *M. trichosporium* OB3b. σ^{70} promoter (Pσ^{70}) regions are indicated in the gene clusters. The σ^{70} promoters were predicted using BPROM (73).

Also important to the discussion here is that after MB is secreted into the extracellular environment, it is subsequently taken up by a TonB-dependent transporter (TBDT) encoded by *mbnT* that is typically found upstream of *mbnA* (Fig. 1a) (37, 45, 46). Interestingly, it has been found that methanotrophs capable of making MB can also take up copper bound to MBs produced by other methanotrophs, e.g., *M. trichosporium* OB3b can take up copper bound to MB-SB2, and such collection regulates the expression of sMMO and pMMO (47). These data suggest that either the mechanism for MB uptake is general, i.e., MbnT associated with the MB gene cluster of *M. trichosporium* OB3b can take up MB-SB2, or this methanotroph can produce other TBDTs that enable it to collect MB made by other methanotrophs.

More detailed knowledge of the mechanism of copper uptake, particularly via MB in methanotrophs, is important given how copper affects methanotrophic activity. Such data would particularly help refine our understanding of how expression of key enzymes in methanotrophs are regulated and provide insight into the mechanism(s) of methanotrophic interactions, i.e., competition for copper. Such information would enhance our ability to manipulate these microbes for valorization of methane, better predict their activity *in situ*, and enable more advanced earth systems models of greenhouse gas emissions. Here, we investigated the function of multiple TBDTs in *M. trichosporium* OB3b in MB uptake and the involvement of these TBDTs in the copper switch.

RESULTS

Bioinformatic and transcriptional analyses of select TBDTs in *M. trichosporium* OB3b. The genome of *M. trichosporium* OB3b encodes 30 TBDTs (Table S1 in the supplemental material), including the TBDT responsible for uptake of MB-OB3b (here labeled MbnT1). One of the other TBDTs, designated here as MbnT2, with gene locus tag ADVE02_v2_10210, has high identity (57.36%) and similarity (E value, 0.0) with MbnT from *Methylocystis* sp. strain SB2 (Table S1). Further, immediately upstream of *mbnT2* are two other genes commonly associated with *mbnT*s, i.e., *mbnI* and *mbnR*, encoding an extracytoplasmic function sigma factor and a putative membrane sensor, respectively (Fig. 1b). Phylogenetic analysis of known MbnTs shows MbnT2 was related most closely to the MbnTs responsible for uptake of group IIA MBs (e.g., MB-SB2) (Fig. 2). This suggests that MbnT2 may enable *M. trichosporium* OB3b to take up MB-SB2, a phenomenon observed previously in *M. trichosporium* OB3b, but the means by which this occurs are not explained (47).

To explore the function of MbnT2 in MB-SB2 uptake, we first measured the expression of *mbnT1* and *mbnT2* in *M. trichosporium* OB3b wild type grown with different amounts of copper and MBs (Fig. 3). *mbnT1* expression was approximately 5-fold higher when no copper was added (only background levels of copper, ~30 nM) versus the addition of 1 μM copper ($0.01 < P < 0.05$). Further, the addition of 5 μM of either form of MB in the presence of 1 μM copper did not significantly affect *mbnT1*
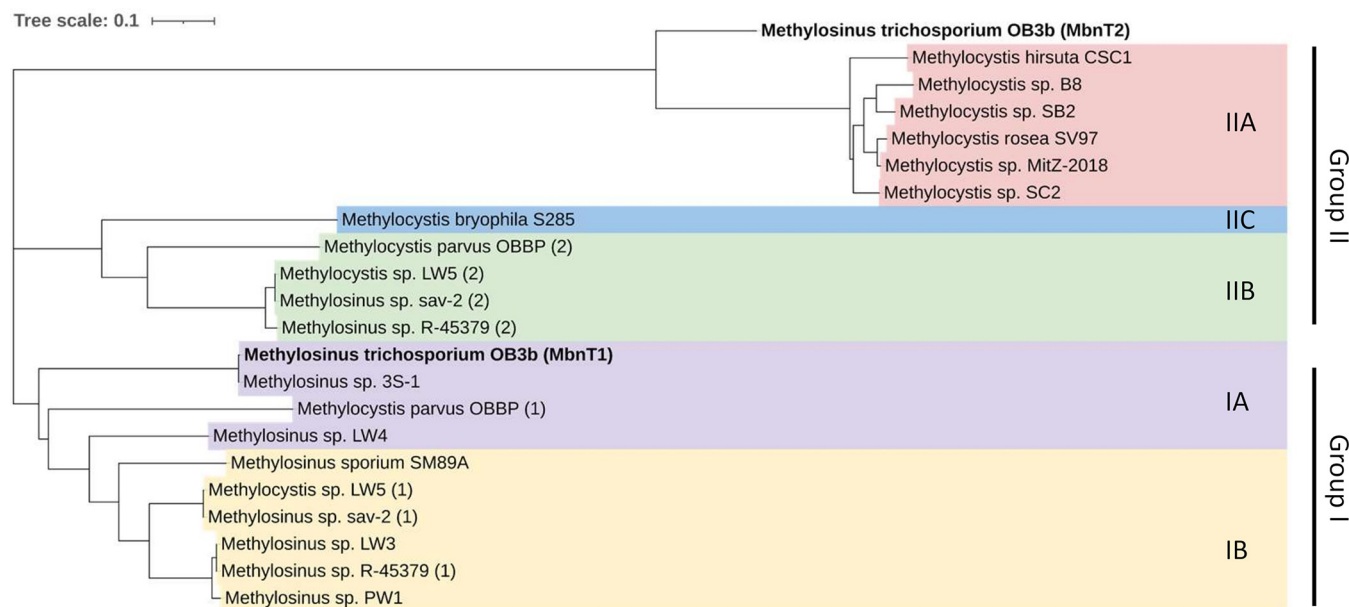
FIG 2 Phylogenetic analysis of the amino acid sequence of putative MbnTs for methanobactin uptake. MbnTs from different groups of methanobactin are indicated with different colors. MUSCLE sequence alignment and the phylogenetic tree construction was processed using Geneious software with default settings. Further polishing of the phylogenetic tree was performed on the Interactive Tree of Life web browser (http://itol.embl.de/). The scale bar represents number of amino acid changes per site.

expression compared to that observed in the presence of 1 μM copper only (Fig. 3a). Like *mbnT1*, *mbnT2* was also upregulated when no copper was added versus in the presence of 1 μM added copper ($\sim$3-fold; $0.01 < P < 0.05$). As was found for *mbnT1*, the addition of 5 μM MB-OB3b in the presence of 1 μM copper did not affect *mbnT2*
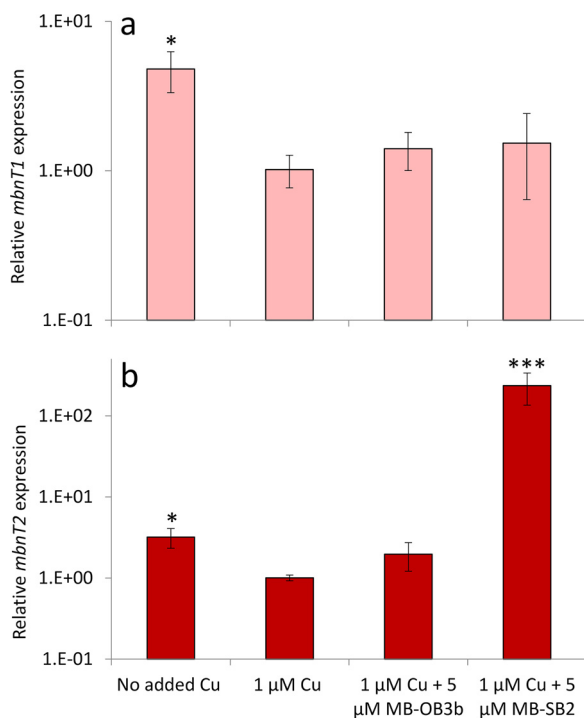
FIG 3 RT-qPCR analysis of the relative expression of *mbnT1* (a) and *mbnT2* (b) in *M. trichosporium* OB3b wild type grown in the absence of added copper, with 1 μM added copper, and with 1 μM added copper plus either 5 μM MB-OB3b or MB-SB2. Error bars indicate standard deviations from triplicate biological cultures. Student's *t* test was performed to detect significant differences between expression in the presence of 1 μM added copper versus other growth conditions, *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.
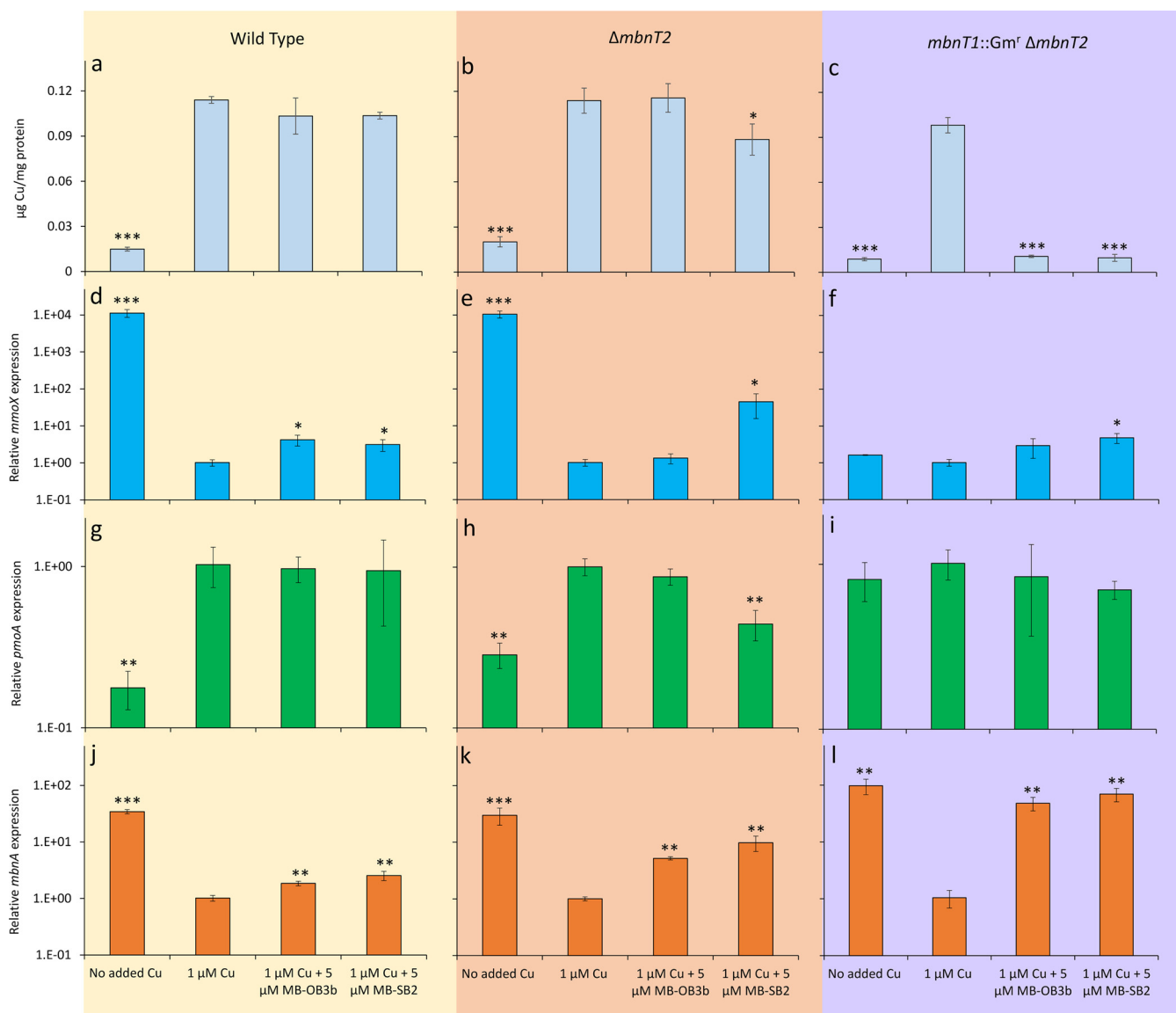
FIG 4 Copper associated with biomass (a to c) and RT-qPCR analysis of the relative expression of *mmoX* (d to f) *pmoA* (g to i), and *mbnA* (j to l) in *M. trichosporium* OB3b wild type (left panels), Δ*mbnT2* mutant (middle panels), and *mbnT1*::Gm^r Δ*mbnT2* mutant (right panels) grown in the absence of added copper, with 1 μM added copper, and with 1 μM added copper plus either 5 μM MB-OB3b or MB-SB2. Error bars indicate standard deviations from triplicate biological cultures. Student's *t* test was performed to detect significant differences between growth in the presence of 1 μM added copper versus other growth conditions. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

expression. When *M. trichosporium* OB3b was grown with 1 μM copper plus 5 μM MB-SB2, however, *mbnT2* was significantly upregulated ($\sim$380-fold; $P < 0.001$) compared to the presence of 1 μM copper only (Fig. 3b).

Deletion of TonB-dependent uptake systems in *M. trichosporium* OB3b. To verify the putative function of MbnT2 in MB-SB2 uptake by *M. trichosporium* OB3b, a markerless deletion of *mbnT2* was constructed (Δ*mbnT2* mutant). *mbnT2* was also deleted in the previously constructed *M. trichosporium mbnT1*::Gm^r mutant (46) to create a double mutant defective in both MbnT1 and MbnT2 (*mbnT1*::Gm^r Δ*mbnT2* mutant). The absence of *mbnT2* and the disruption of *mbnT1* was confirmed by PCR (Fig. S1) and sequencing (data not shown).

Characterization of copper uptake and expression of methane monooxygenases and methanobactin production in *M. trichosporium* OB3b wild-type and Δ*mbnT2* strains. Both *M. trichosporium* OB3b wild-type and the Δ*mbnT2* mutant strains had significantly greater copper associated with biomass when grown with 1 μM versus no added copper (Fig. 4a). There was no significant difference in the amount of copper associated

with biomass for either strain when grown with 1 μM copper with 5 μM MB-OB3b versus 1 μM copper alone (Fig. 4a and b). Biomass-associated copper was also not significantly different for *M. trichosporium* OB3b wild type grown with 1 μM copper plus 5 μM MB-SB2 versus 1 μM copper alone, but did decrease slightly (~20%; 0.01 < *P* < 0.05) in the *M. trichosporium* Δ*mbnT2* mutant.

sMMO expression is commonly monitored via reverse transcription-quantitative PCR (RT-qPCR) of *mmoX* (encoding the α-subunit of the hydroxylase component of sMMO). *mmoX* expression was over 4 orders of magnitude greater when no copper was added versus 1 μM copper for both wild-type and Δ*mbnT2* mutant strains (*P* < 0.001) (Fig. 4d and e). *mmoX* expression slightly increased in *M. trichosporium* OB3b wild type in the presence of 1 μM copper and 5 μM of either form of MB (an increase of 3 to 4×; 0.01 < *P* < 0.05), but only increased in the presence of 1 μM copper plus 5 μM MB-SB2 for the Δ*mbnT2* mutant of *M. trichosporium* OB3b (~45-fold; 0.01 < *P* < 0.05) (Fig. 4e). The naphthalene assay showed no observable sMMO activity of the Δ*mbnT2* mutant cells grown with 1 μM copper and 5 μM of either form of MB (Fig. S2).

Similarly, pMMO expression is commonly monitored via RT-qPCR of *pmoA* (encoding the β-subunit of pMMO). *pmoA* expression was ~5.8-fold-higher when 1 μM copper was added versus no copper addition and was not significantly affected with the concomitant addition of 5 μM of either MB for *M. trichosporium* OB3b wild type (Fig. 4g). Similarly, expression of *pmoA* increased ~4-fold (0.01 < *P* < 0.05) with 1 μM versus no added copper for the *M. trichosporium* OB3b Δ*mbnT2* mutant, and such expression did not significantly change with the addition of MB-OB3b (Fig. 4h). *pmoA* expression, however, decreased by ~55% (0.001 < *P* < 0.01) in the presence of 1 μM copper plus 5 μM MB-SB2 compared to 1 μM copper alone in the *M. trichosporium* OB3b Δ*mbnT2* mutant (Fig. 4g and h).

mbnA expression increased ~35-fold (*P* < 0.001) when no copper was added versus in the presence of 1 μM added copper for *M. trichosporium* OB3b wild type (Fig. 4j). The addition of 5 μM of either form of MB in the presence of copper also increased *mbnA* expression in *M. trichosporium* OB3b wild type compared to the addition of copper alone (1.8 to 2.5×; 0.001 < *P* < 0.01). Expression of *mbnA* in *M. trichosporium* OB3b Δ*mbnT2* similarly was ~30× higher when no copper was added versus in the presence of 1 μM added copper (*P* < 0.001; Fig. 4k). Further, *mbnA* expression increased in *M. trichosporium* OB3b Δ*mbnT2* in the presence of 1 μM copper and 5 μM of either form of MB compared to copper alone (0.001 < *P* < 0.01), but expression increased more in the presence of MB-SB2 (~10×) versus MB-OB3b (~5×).

Characterization of copper uptake and expression of methane monooxygenases and methanobactin production in *M. trichosporium* OB3b *mbnT1*::Gm^r Δ*mbnT2*. To further delineate the mechanism(s) underlying MB uptake by *M. trichosporium* OB3b, we constructed and characterized the double mutant of *mbnT1*::Gm^r Δ*mbnT2*. Copper associated with biomass when no additional copper was provided was an order of magnitude less than in the presence of 1 μM copper for this mutant (~12-fold less, *P* < 0.001). Copper uptake was similarly reduced by an order of magnitude when 5 μM of either form of MB was provided in addition to 1 μM copper compared to copper alone (*P* < 0.001) (Fig. 4c). Further, the addition of 1 μM copper had little effect on either *mmoX* or *pmoA* expression in this double mutant compared to when no copper was added (Fig. 4f and i). Expression of *mmoX* increased ~3× when 5 μM MB-OB3b was added in conjunction with 1 μM copper, but this was not significantly different from when only copper was added (*P* > 0.05). *mmoX* expression in the *mbnT1*::Gm^r Δ*mbnT2* mutant increased more when 5 μM MB-SB2 was added in conjunction with copper and was significantly different from when only copper was added (~5×; 0.01 < *P* < 0.05). *pmoA* expression was not affected by the addition of either form of MB in the presence of 1 μM copper. Collectively, these data indicate that the copper switch was disrupted in this strain, i.e., *mmo* and *pmo* gene expression did not respond to the addition/nonaddition of copper. Finally, expression of *mbnA* was 2 orders of magnitude greater (0.001 < *P* < 0.01) when no copper was added than in the
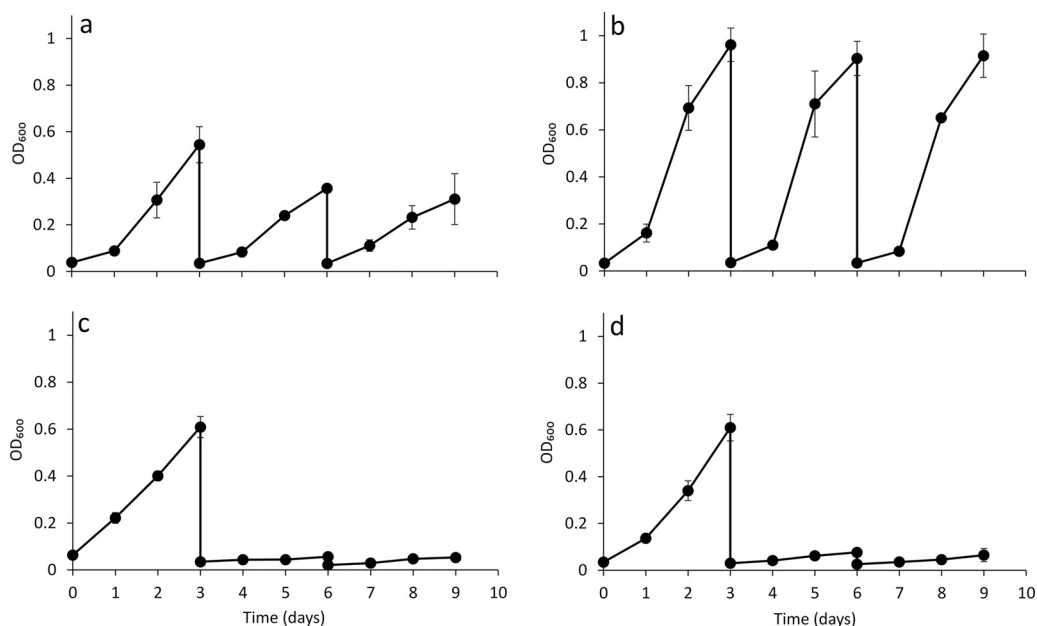
FIG 5 Growth of *M. trichosporium* mbnT1::Gmʳ ΔmbnT2 mutant in the absence of added copper (a), with 1 μM added copper (b), with 1 μM added copper plus 5 μM MB-OB3b (c), or with 1 μM added copper plus 5 μM MB-SB2 (d). Error bars indicate standard deviations from triplicate biological cultures.

presence of 1 μM added copper in this double mutant (Fig. 4l). Cultivation of this mutant in the presence of 1 μM copper plus 5 μM of either form of MB increased *mbnA* expression by ~50 to 70× compared to the presence of copper alone ($0.001 < P < 0.01$) (Fig. 4l).

Further growth experiments confirmed that of mbnT1::Gmʳ ΔmbnT2 mutant of *M. trichosporium* OB3b was likely unable to carefully regulate expression of the alternative forms of MMO. That is, growth of this double mutant in methane was observed if transferred multiple times without extra copper addition or in the presence of 1 μM added copper (Fig. 5a and b). sMMO activity, however, was not apparent via the naphthalene assay when copper was not added when characterized at the end of the third cycle (Fig. S3). If this mutant was grown in the presence of 1 μM copper and 5 μM of either form of MB, however, growth stopped after a second sequential transfer (Fig. 5c and d). These data suggest that this mutant is unable to collect copper in the presence of either form of MB but does have some mechanism(s) to scavenge trace amounts of copper in their absence. Growth, however, was inhibited when no copper was added compared to growth in the presence of 1 μM copper, i.e., the maximum cell density of the mbnT1::Gmʳ ΔmbnT2 mutant of *M. trichosporium* OB3b grown with only background levels of copper (~30 nM) was ~30% of the biomass when grown with 1 μM copper (Fig. 5a and b). It also appears, based on RT-qPCR and sMMO activity assays, that this mutant is unable to effectively utilize sMMO for methane oxidation, especially when copper is completely unavailable (i.e., through exogenous provision of MB) and instead constitutively expresses (and solely relies on) pMMO activity for growth.

These results indicate that in the *M. trichosporium* OB3b mbnT1::Gmʳ ΔmbnT2 mutant, the copper switch was disrupted. To investigate this further, both *M. trichosporium* OB3b wild-type and mbnT1::Gmʳ ΔmbnT2 mutant strains were grown with methanol (0.5%) as the growth substrate and various amounts of copper (either no addition or 1 μM). As observed earlier (18), the copper switch was clearly apparent in *M. trichosporium* OB3b wild type when grown in the presence of methanol with and without extra addition of 1 μM copper (Fig. S4a, c, and e). Specifically, *mmoX* expression and sMMO activity clearly responded to the addition/nonaddition of copper in the wild-type strain when grown with methanol (Fig. S4a and e). Conversely, as was found for methane-grown cultures,

expression of *pmoA* in *M. trichosporium* OB3b wild type increased by approximately 8-fold when 1 μM copper was added versus no copper (0 μM) addition. When the *M. trichosporium* OB3b *mbnT1*::Gm^r $\Delta mbnT2$ mutant strain was grown on methanol and various amounts of copper, again, as found for methane-grown cultures, *mmoX* expression was invariant with respect to copper, and the naphthalene assay did not indicate any sMMO activity under any condition (Fig. S4b and f). *pmoA* expression for methanol-grown cultures of *M. trichosporium* OB3b *mbnT1*::Gm^r $\Delta mbnT2$, however, did increase ~3× when grown in the presence of 1 μM copper versus no added copper (Fig. S4d). Such a difference was significant ($P = 0.013$) but less than that observed in *M. trichosporium* OB3b wild type grown on methanol (where *pmoA* expression increased ~8× in the presence of 1 μM copper versus no added copper; $P = 0.0007$) (Fig. S4c).

DISCUSSION

MBs have been traditionally categorized into one of two general groups based on general chemical composition and structure. Recent phylogenetic analyses suggest that these groups can be further subdivided into at least five smaller groups, i.e., groups IA, IB, IIA, IIB, and IIC, although what, if any, structural differences that may exist between these subgroups is unclear, as MBs to date have only been characterized from groups IA and IIA (37). Our phylogenetic analyses of TBDTs involved in MB uptake (MbnTs), however, suggest that these also can generally be divided into groups that correspond to the proposed MB subgrouping system, suggesting that these MB subgroups may indeed have different structures (Fig. 2). That is, the general correspondence of grouping of the genes involved in MB biosynthesis with MB uptake suggests that MbnTs preferentially bind and import specific forms of MB due to different structures these MBs make after binding copper. Such a phenomenon is quite possible, as it has been shown that different siderophores are taken up preferentially by different TBDTs (48, 49).

Previous studies indeed demonstrated MbnT1 encoded by the *mbn* gene cluster of *M. trichosporium* OB3b is the only TBDT responsible for MB-OB3b uptake (45, 46). Here, we show, using various mutants, that MbnT2 preferentially binds and transports MB-SB2 (a group IIA MB), but we cannot exclude, at this time, that MB-SB2 may also be collected to some degree by *M. trichosporium* OB3b via MbnT1. That is, although copper uptake was slightly yet significantly reduced (~20%) in the *M. trichosporium* OB3b $\Delta mbnT2$ mutant in the presence of MB-SB2, it was still apparent. Such a hypothesis is supported by the finding that copper uptake was effectively abolished in the double mutant of *M. trichosporium* OB3b *mbnT1*::Gm^r $\Delta mbnT2$ in the presence of either MB-OB3b or MB-SB2. Such a conclusion, however enticing, must be treated with some caution, as both the *M. trichosporium* OB3b $\Delta mbnT2$ and *M. trichosporium* OB3b *mbnT1*::Gm^r $\Delta mbnT2$ mutants are still able to produce MB-OB3b, as expression of the biosynthetic genes for MB production is under the control of a separate promoter from *mbnT1* (Fig. 1). An alternative explanation may be that MB-OB3b can extract copper to some degree from MB-SB2, thus allowing for some copper uptake by *M. trichosporium* OB3b $\Delta mbnT2$ via *mbnT1* in the presence of copper and MB-SB2, but it was reduced compared to the presence of copper only. We cannot reject either hypothesis at this time, and more effort is clearly needed to address this point.

As found before, expression of MB biosynthesis and uptake genes (i.e., *mbnA*, *mbnT1*, and *mbnT2*) were regulated by the availability of copper, with expression decreasing when copper was added versus no added copper (41). Such a finding is logical, for, as copper availability increases, the need to express high-affinity systems such as MB to collect copper is relaxed. Interestingly, here, we find that *mbnT1* expression in *M. trichosporium* OB3b was not significantly affected by the presence of either MB-OB3b or MB-SB2, but *mbnT2* expression dramatically increased in the presence of MB-SB2 and copper. This suggests that *M. trichosporium* OB3b has an active mechanism to sense and collect MBs made by other methanotrophs. Such a possibility is not unprecedented, as many microorganisms have been found to take up siderophores to satisfy iron requirements,

and the systems required for such uptake are often induced by the presence of heterologous siderophores (50–57).

In addition to participating in uptake of copper in the presence of MBs, it appears that MbnT1 and MbnT2 collectively play a very important role in the canonical copper switch in *M. trichosporium* OB3b. That is, it was earlier shown that mutants of *M. trichosporium* OB3b, either defective in producing MB or in its uptake (i.e., disruption of *mbnA* or *mbnT*, respectively), still differentially expressed sMMO and pMMO, as copper availability was varied (34, 46). If *mbnT2* was deleted, again, the copper switch was apparent when no copper was added versus in the presence of 1 μM added copper (Fig. 4). When both *mbnT1* and *mbnT2* were knocked out, however, the copper switch was disrupted in that *mmoX* expression in this mutant did not respond to copper, and activity of sMMO was never apparent, even when copper was not added for both methane and methanol-grown cultures of the *M. trichosporium* OB3b *mbnT1*::Gm^r $\Delta mbnT2$ mutant (Fig. 4; Fig. S3 and S4). Further, *pmoA* expression also did not respond to the addition of 1 μM copper in this mutant when grown on methane (Fig. 4), but there was some response for methanol-grown cultures when 1 μM copper was added. However, such an increase was much less than that observed in *M. trichosporium* OB3b wild type.

It should be noted that multiple regulatory models for the copper switch have been proposed. One presumes that the switch is based on a FecIRA-like signal cascade similar to that found in siderophore synthesis. That is, as shown in Fig. 1, both *mbnT1* and *mbnT2* are part of a FecIRA-like gene cluster where immediately upstream of these *mbnTs* are genes encoding a putative membrane sensor (*mbnR*) and an extracytoplasmic sigma factor (*mbnI*). In many siderophore production systems, after an outer membrane transporter such as TBDT binds a ferrisiderophore, a signal cascade is observed where the outer membrane transporter transmits a signal to the membrane sensor that activates the extracytoplasmic sigma factor, subsequently inducing further siderophore production (and in some cases, other unrelated genes) (58, 59). Given the strong similarity in gene organization between MB and siderophore uptake systems, it has been proposed that an analogous regulatory system exists in methanotrophs that express MB (42). Such a model, although certainly appropriate for iron-regulated gene expression in some microbes, is not supported by previous data that showed disruption of *mbnT1* did not affect the copper switch (46). Rather, the original model proposed for the copper switch is that it is based on MmoD, a polypeptide encoded within the *mmo* operon of those methanotrophs that exhibit the copper switch and that MB (and any signal cascade associated with its uptake) serves to amplify the magnitude of the switch (34).

The data presented here provide the basis of a third model that may reconcile these conflicting proposals for the copper switch. That is, when MB uptake was prevented through the knockout of both *mbnT1* and *mbnT2*, the copper switch was clearly disrupted. It thus appears that in *M. trichosporium* OB3b, the copper switch has multiple layers of control, i.e., *M. trichosporium* OB3b has redundant mechanisms to collect copper via MB and TBDT (MbnT1 and MbnT2), and only if both are disrupted is the copper switch no longer operative. It thus appears that one TBDT for MB uptake, along with associated regulatory genes (i.e., *mbnI* and *mbnR*), is sufficient to maintain the copper switch in conjunction with MmoD. Given these new data, we present an updated model for the copper switch in Fig. 6. Here, it is proposed that in the absence of significant amounts of copper, MmoD represses *pmo* gene expression, while the binding of copper-free MB to MbnT activates MbnI that upregulates expression of known regulatory elements of the *mmo* operon (MmoR and MmoG). This is possible, as it has been found that iron-free siderophores can be bound to specific TBDTs, inducing gene expression (55, 60). MmoR and MmoG then, in conjunction with MB, may increase expression of genes encoding various sMMO polypeptides as well as MmoD. Further, MbnI, along with MmoD, is proposed to upregulate expression of *mbn* genes. When copper is present, copper-MB complexes can be taken up that inhibit the ability of
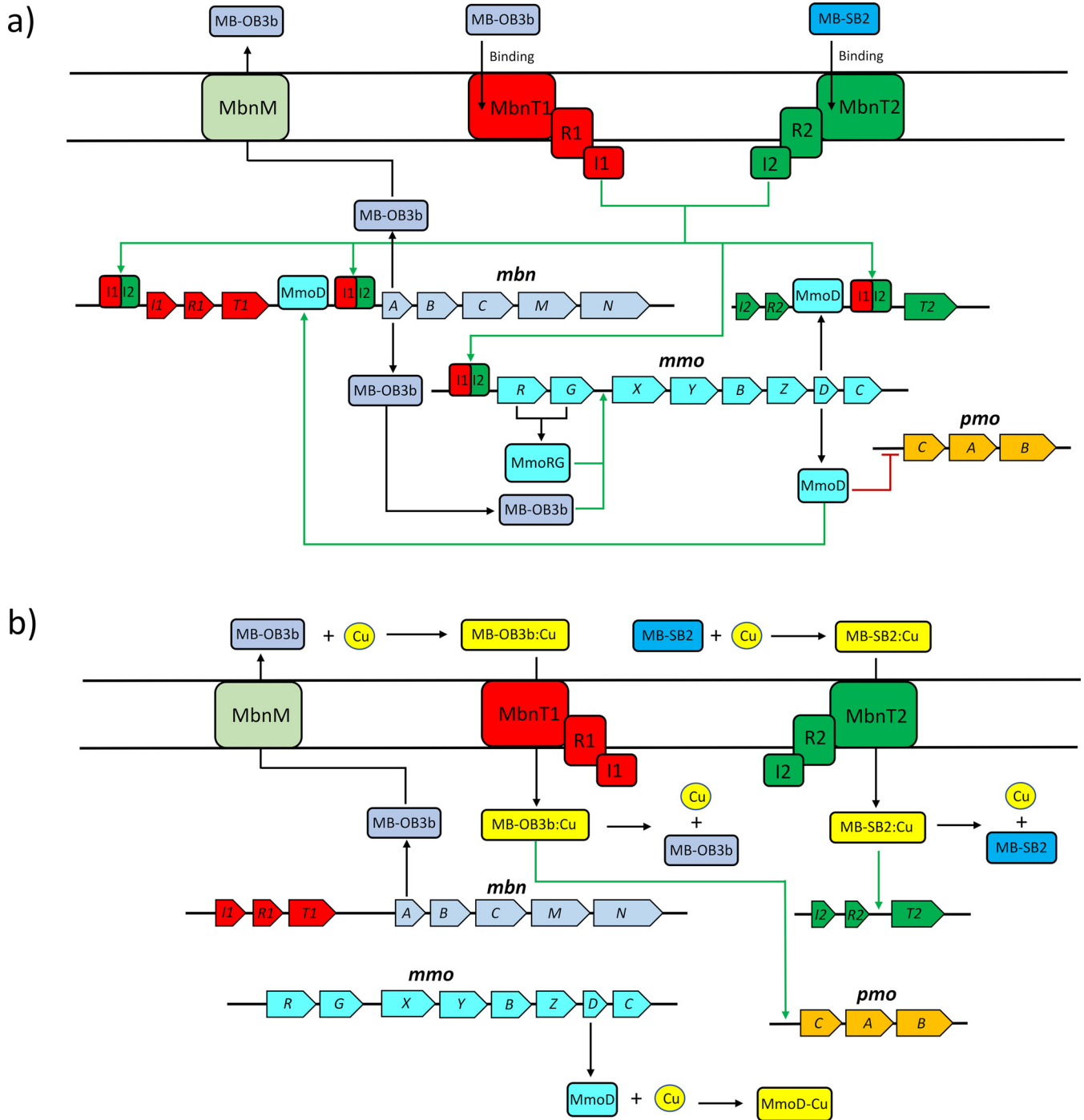
FIG 6 Proposed regulatory scheme of *mmo*, *pmo*, and *mbn* operons in *M. trichosporium* OB3b grown in the absence of added copper (a) or with copper plus MB-OB3b or copper plus MB-SB2 (b). Red lines and green arrows indicate repression or promotion of the gene transcription.

signal transduction from MbnT through MbnR to MbnI, and thus, expression of *mbn* and *mmo* genes is reduced. After copper-MB uptake, copper is released and associated with MmoD that then serves to prevent repression of the *pmo* operon. In addition, based on earlier findings, copper-MB complexes may also serve to enhance expression of the *pmo* operon, but this is not critical for such expression (34).

Such a model is intriguing, as it indicates that methanotrophs have not only evolved high-affinity means to collect copper (i.e., MB), but at least one (*M. trichosporium* OB3b) has developed a unique regulatory system to recognize and actively take

TABLE 1 Bacterial strains used in this study

Strain	Description and/or genotype	Reference or source
Methylocystis sp. strain SB2	Wild type	74
M. trichosporium OB3b	Wild type	
M. trichosporium mbnT1::Gm^r	*mbnT1* partially exchanged with gentamicin resistance gene	46
M. trichosporium ΔmbnT2	*mbnT2* deleted	This work
M. trichosporium mbnT1::Gm^r ΔmbnT2	*mbnT1* partially exchanged with gentamicin resistance gene, and *mbnT2* deleted	This work
E. coli TOP10	Strain used for plasmid construction and cloning. F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
E. coli S17-1	Conjugative donor. *recA1 thi pro hsdR*-RP4-2Tc::Mu Km::Tn7	67

up MB produced by another methanotroph by upregulating the system required for its uptake. Upregulation of *mbnT1* was not observed when additional MB-OB3b was provided, suggesting that there are some critical, as-yet-unknown regulatory differences between MbnT1/T2, MbnR1/R2, and/or MbnI1/I2 to enable *M. trichosporium* OB3b to selectively upregulate *mbnT2* in the presence of MB-SB2 but not *mbnT1* in the presence of MB-OB3b. It is thus still unclear how *M. trichosporium* OB3b is able to differentially respond to different forms of MB. An additional issue that should be considered is that although our data indicate that specific TBDTs target different forms of MB to take up, we cannot exclude the possibility that MbnIR associated with one MbnT cannot form an effective complex with the other to enable a signal cascade to be created. Such a possibility is intriguing, as it would again indicate that methanotrophs that exhibit the copper switch have multiple layers of control to ensure appropriate expression of MMOs dependent on copper availability.

In conclusion, we show that MB is important for copper collection in methanotrophs, and some individuals are not only able to produce and collect their own form of MB but also have evolved specific machinery and regulatory systems to actively take up MB from others. Such character may have two purposes, i.e., it would enable the recipient to (i) capture copper bound to various forms of MB that are usually unavailable for many microorganisms, including methanotrophs and denitrifiers (61, 62); and (ii) improve its competitiveness by placing other MB-producing methanotrophs at a disadvantage. These studies also expand our understanding of how methanotrophs interact by elucidating that different forms of MB require different uptake systems and that at least one methanotroph utilizes multiple MB uptake systems to create substantial redundancy in the copper switch that plays a critical role in its metabolism. Such information has significant potential for enhancing future efforts aimed at manipulating methanotrophs for a number of lucrative and important purposes.

MATERIALS AND METHODS

Methanobactin isolation. Methanobactin (MB) from *M. trichosporium* OB3b and *Methylocystis* sp. strain SB2 were isolated from their spent media as previously described by Bandow et al. (63). The purity of the isolated methanobactins was determined by high-performance liquid chromatography (HPLC) as described earlier (64).

Growth conditions. *Methylosinus trichosporium* OB3b and constructed mutants (Table 1) were grown in nitrate mineral salt (NMS) medium (6) with or without 1 μM CuCl$_2$. For NMS medium without added copper, the background copper concentration was found to be 30 $\pm$ 10 nM. Methane and air were added at a methane-to-air ratio of 1:2. For methanol grown cultures, 0.5% (vol/vol) methanol was added to the NMS medium as described before (18). Cultures were incubated in the dark at 30°C. Liquid cultures were grown in 250-ml sidearm Erlenmeyer flasks with 20 to 30 ml NMS medium with shaking at 200 rpm. Methanobactin from *M. trichosporium* OB3b or *Methylocystis* sp. strain SB2 were filter sterilized and added to NMS medium at a final concentration of 5 μM as described earlier (65). Solid NMS medium was supplemented with 1.2% agar. Growth was monitored by measuring the optical density at 600 nm (OD$_{600}$) with a Genesys 20 visible spectrophotometer (Spectronic Unicam, Waltham, MA). Triplicate biological cultures were harvested at mid- to late-exponential phase for OD$_{600}$ measurement, transcriptional analysis of specific gene expression, and metal distribution. *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C with or without supplementation of 25 μg/ml kanamycin.

Construction of *M. trichosporium* OB3b mutants. *mbnT2* was knocked out using a previously described protocol (66) with modifications. Briefly, upstream and downstream regions of *mbnT2* (arms A

TABLE 2 Primers used in this study

Targeted gene	Primers (sequence [5′–3′])[a]	Application	Reference or source
mbnT1	mbnT1-checking_F (CCGATCGAACCTGGCTCTAT), mbnT1-checking_R (ATTGTAAATCGTGACGGCGG)	PCR	46
mbnT1	qmbnT1_F (TATGCGCCGGTCTATGGTTC), qmbnT1_R (GCCGAGATCATGTCCTGGAG)	RT-qPCR	This work
mbnT2	mbnT2_F (CTGAAGACCGTGAATCCGCT), mbnT2_R (GTCCATTGGCCTGTGTGAGA)	PCR	This work
mbnT2	mbnT2-armA_F, (ATTTTT gaattc ACATGAGCAACGGCCAGAAA)[b], mbnT2-armA_R (ATTTTT cactttgtg GAACGCGGAACCTCCTTCA)[b]	PCR	This work
mbnT2	mbnT2-armB_F (ATTTTT cacaaagtg TGAATGGTTCCGCAACGAGA)[b], mbnT2-armB_R (ATTTTT aagctt AGAAAAGGCCGCCTACCTTC)[b]	PCR	This work
mbnT2	qmbnT2_F (GCAATATAGTCCCGGCGTGT), qmbnT2_R (AGAAAGGGTATGTCGTGCCG)	RT-qPCR	This work
pmoA	pmoA_F (TTCTGGGGGCTGGACCTAYTTC), pmoA_R (CCGACAGCAGCAGGATGATG)	RT-qPCR	75
mmoX	mmoX_F (TCAACACCGATCTSAACAACG), mmoX_R (TCCAGATTCCRCCCCAATCC)	RT-qPCR	75
mbnA	mbnA-checking_F (GCGATCAAGTAGGTATAACTTGGAA), mbnA-checking_R (CAATTCCTCCCGATCTCTTTC)	PCR	39
mbnA	qmbnA_F (TGGAAACTCCCTTAGGAGGAA), qmbnA_R (CTGCACGGATAGCACGAAC)	RT-qPCR	34
16S rRNA	Eub_341F (CCTACGGGAGGCAGCAG) Eub_534R (ATTACCGCGGCTGCTGGC)	RT-qPCR	76

[a]Y, S, and R are the IUPAC DNA codes for the C/T, C/G, and A/G nucleobases, respectively.
[b]Lowercase letters indicate EcoRI, AdeI, or HindIII restriction site sequences included in these primers.

and B, respectively) were PCR amplified using the arm primers listed in Table 2. Arms A and B were digested with the restriction enzymes and ligated together to form armAB, which was subsequently inserted into pK18mob*sacB* mobilizable suicide vector (Fig. S5) (67). The pK18mob*sacB* vector with armAB was transferred to *E. coli* TOP10 (Invitrogen, Carlsbad, CA). Plasmid was extracted from transformed *E. coli* Top10 using the plasmid minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted plasmid was then transferred to *Escherichia coli* S17-1 (68). Conjugation of *E. coli* S17-1 carrying the constructed vector with *M. trichosporium* OB3b was performed as described by Martin and Murrell (69). Transconjugants were grown on NMS plates supplemented with 25 μg/ml kanamycin and 10 μg/ml nalidixic acid. One kanamycin-resistant transconjugant colony (generated after 10 days incubation) was transferred to an NMS plate with kanamycin (25 μg/ml) and incubated for 7 days and subsequently transferred to an NMS plate with 2.5% sucrose (mass/vol). Sucrose-resistant colonies were generated after 10 days incubation and were screened for mutant with deletion of *mbnT2* by colony PCR using the checking primers (Table 2). The successful *mbnT2* deletion mutant was further confirmed by PCR with DNA extracted from the mutant using the DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The procedure for construction and confirmation of the *mbnT1*::Gm^r Δ*mbnT2* double mutant was the same as described above, except using *mbnT1*::Gm^r (46) mutant strain instead of the wild-type *M. trichosporium* OB3b for the conjugation step.

RNA isolation and RT-qPCR. RNA isolation was performed with a bead-beating procedure followed by column purification using RNeasy minikit (Qiagen, Hilden, Germany) as described before (70). Genomic DNA was removed on the column with RNase-free DNase (Qiagen, Hilden, Germany) treatment. Absence of genomic DNA was confirmed by 16S rRNA gene-targeted PCR with extracted RNA samples as the templates. The purified RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized from 200 ng total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

RT-qPCR was performed to determine the relative expression of the *mbnT1*, *mbnT2*, *pmoA*, *mmoX*, and *mbnA* in *M. trichosporium* OB3b and mutant strains grown with and without addition of copper and/or methanobactins. Primers for amplification of the two *mbnT* genes were designed using the NCBI online primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificity was checked with the online tool and further verified by PCR, gel electrophoresis, and sequencing. RT-qPCR was performed using the iTaq Universal SYBR green supermix (Bio-Rad, Hercules, CA) with 96-well PCR plates on a CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA). The RT-qPCR program was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s. Melting curves were measured from 65°C to 95°C with increments of 0.5°C and 10 s at each step. Transcription of the targeted genes was determined using cDNA as the template. The transcript levels were calculated by relative quantification using the quantification cycle ($2^{-\Delta\Delta Cq}$) method (71) with the 16S rRNA gene as the reference gene (72).

Metal analysis. Cells of *M. trichosporium* OB3b and the mutant strains grown under different conditions were collected and acid digested as described early (46, 72). Copper associated with biomass was subsequently analyzed using an inductively coupled plasma mass spectrometer (ICP-MS; Agilent Technologies, Santa Clara, CA).

Data availability. Materials and data generated in this study will be made available upon reasonable request to the corresponding author.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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