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A TonB-Dependent Transporter Is Responsible for Methanobactin Uptake by *Methylosinus trichosporium* OB3b

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Methanobactin, a small modified polypeptide synthesized by methanotrophs for copper uptake, has been found to be chromosomally encoded. The gene encoding the polypeptide precursor of methanobactin, *mbnA*, is part of a gene cluster that also includes several genes encoding proteins of unknown function (but speculated to be involved in methanobactin formation) as well as *mbnT*, which encodes a TonB-dependent transporter hypothesized to be responsible for methanobactin uptake. To determine if *mbnT* is truly responsible for methanobactin uptake, a knockout was constructed in *Methylosinus trichosporium* OB3b using marker exchange mutagenesis. The resulting *M. trichosporium mbnT*::Gm^r mutant was found to be able to produce methanobactin but was unable to internalize it. Further, if this mutant was grown in the presence of copper and exogenous methanobactin, copper uptake was significantly reduced. Expression of *mmoX* and *pmoA*, encoding polypeptides of the soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO), respectively, also changed significantly when methanobactin was added, which indicates that the mutant was unable to collect copper under these conditions. Copper uptake and gene expression, however, were not affected in wild-type *M. trichosporium* OB3b, indicating that the TonB-dependent transporter encoded by *mbnT* is responsible for methanobactin uptake and that methanobactin is a key mechanism used by methanotrophs for copper uptake. When the *mbnT*::Gm^r mutant was grown under a range of copper concentrations in the absence of methanobactin, however, the phenotype of the mutant was indistinguishable from that of wild-type *M. trichosporium* OB3b, indicating that this methanotroph has multiple mechanisms for copper uptake.

Methanotrophs, or methane-oxidizing bacteria, are a group of microbes with great environmental and industrial importance. For example, methanotrophs are well known to play a key role in controlling the net emission of methane from soils, a potent greenhouse gas with a global warming potential of ~34 times that of carbon dioxide over a 100-year time frame (1). In fact, it is estimated that as much as 90% of methane generated in anaerobic soils via methanogenesis may be removed via methanotrophy (2). Further, methanotrophs oxidize methane under ambient temperatures and pressures and, thus, are attractive platforms for the valorization of methane to products such as single-cell protein, bioplastics, biofuels, and osmoprotectants (3–5).

Methanotrophs are fairly ubiquitous and are found in many different environments, including forest soils, landfill cover soils, agricultural soils, freshwater and marine sediments, and many other locations (4, 6, 7). Although methane oxidation is commonly associated with oxygen reduction, in the past 15 years, methane oxidation has also been shown to be coupled with sulfate, nitrite, and nitrate reduction (4, 8–10). Methanotrophs also show remarkable phylogenetic diversity, with aerobic methanotrophs grouping in the *Gammaproteobacteria* and *Alphaproteobacteria* as well as in the NC10 and *Verrucomicrobia* phyla (4, 6, 8).

A key issue affecting aerobic methanotrophic activity, particularly the activities in the *Gammaproteobacteria* and *Alphaproteobacteria*, is the availability of copper. It was first discovered >30 years ago that some methanotrophs exhibited a unique "copper switch," where the form and activity of the methane monooxygenase (MMO) dramatically changes with changing copper availability. Specifically, it was found that under copper-limiting conditions, some methanotrophs synthesized a cytoplasmic or soluble methane monooxygenase (sMMO). As copper levels increased, expression of sMMO decreased, while expression and activity of a membrane-bound or particulate methane monooxygenase (pMMO) increased (4, 11, 12). The sMMO has a broad substrate range and, as a result, has great versatility for use in biocatalysis and bioremediation, but it also has a relatively poor affinity for methane (4, 13–16). pMMO, conversely, has a relatively narrow substrate range and a greater specificity for methane, suggesting that strategies to utilize methanotrophs to reduce methane emissions and/or remove methane from the atmosphere should target pMMO-expressing methanotrophs (14, 17, 18).

The mechanism underlying this copper switch was recently found to involve a novel copper-binding compound or chalkophore called methanobactin. Methanobactin is a small, modified polypeptide (<1,200 Da) with two heterocyclic rings, either an imidazole, an oxazolone, or a pyrazinedione ring, each with an

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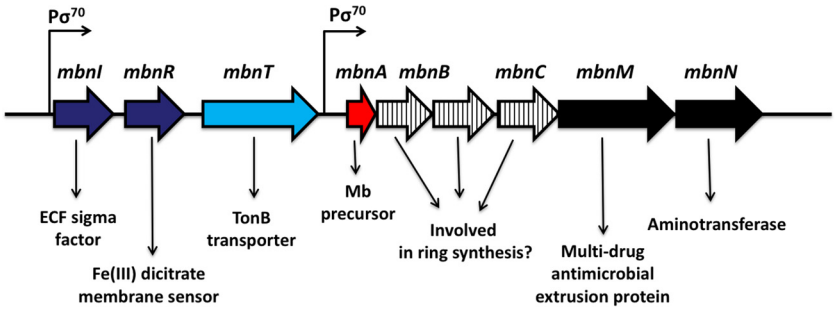

FIG 1 Methanobactin gene cluster in *Methylosinus trichosporium* OB3b. ECF, extracytoplasmic function; Mb, methanobactin.

associated enethiol group, that together are responsible for copper binding (19–22). Biochemical analyses indicated that methanobactin may be formed from a polypeptide precursor with the heterocyclic rings derived from an -X-Cys dipeptide sequence (22). Interrogation of available methanotrophic genomes found one possible candidate gene, *mbnA*. Deletion of *mbnA* in *Methylosinus trichosporium* OB3b showed that it is indeed the precursor of methanobactin and that it is part of a gene cluster (Fig. 1) with many genes of unknown function (possibly involved in methanobactin formation) as well as an aminotransferase (also possibly involved in methanobactin formation) and an extrusion protein (that may serve to secrete methanobactin). Upstream of *mbnA* is a gene encoding a TonB-dependent transporter (*mbnT*) that has been suggested, but not shown, to be involved in methanobactin uptake (23).

To elucidate the role of *mbnT* in methanobactin uptake, we created mutants of *M. trichosporium* OB3b in which *mbnT* has been selectively knocked out via marker exchange mutagenesis.

MATERIALS AND METHODS

Growth conditions. Wild-type *Methylosinus trichosporium* OB3b and the *mbnT*::Gm^r mutant (constructed as described below) were grown on nitrate mineral salt (NMS) medium (24) at 30°C with CH_4 added at a methane-to-air ratio of 1:2. Liquid cultures were grown in 250-ml sidearm Erlenmeyer flasks with 30 to 50 ml of medium shaken at 200 rpm. Copper (as $CuCl_2$) and methanobactin from *M. trichosporium* OB3b were filter sterilized and were added to NMS medium as described earlier (25). Growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a Genesys 20 visible spectrophotometer (Spectronic Unicam, Waltham, MA) at 3- to 12-hour intervals. Cultures were grown in at least duplicate biological replicates and were harvested at late exponential phase for analysis of specific gene expression and metal distribution.

Knockout of *mbnT*. Marker exchange mutagenesis was applied to create a knockout of *mbnT*, which encodes a TonB-dependent transporter using the protocol described in the work of Semrau et al. (23). Briefly, 3′ and 5′ DNA regions of *mbnT* (arms A and B, respectively) were selectively amplified by PCR using the primers listed in Table 1. These PCR products were then digested with BamHI, separated by gel electrophoresis, and purified using the QIAquick gel extraction kit (Qiagen) by following the manufacturer's instructions. Arms A and B were ligated and were again PCR amplified. The amplified product was digested with EcoRI and HindIII and was inserted into pK18mobsacB, yielding the construct pWG01. The gentamicin resistance gene (Gm^r) was then excised from plasmid p34S-Gm using BamHI. This was then inserted into the BamHI site between arms A and B to give the construct pWG011. This was then used to transform *Escherichia coli* S17.1 (26). *E. coli* S17.1 was then conjugated with *M. trichosporium* OB3b as described by Martin and Murrell (27). Transconjugants were identified by plating cells onto NMS plates with 2.5 $\mu g \cdot ml^{-1}$ gentamicin. Residual contamination by *E. coli* S17.1 was then removed by subsequently growing the resulting *mbnT*::Gm^r mutant of *M. trichosporium* OB3b in NMS medium with 2.5 $\mu g \cdot ml^{-1}$ gentamicin and 10 $\mu g \cdot ml^{-1}$ nalidixic acid. Successful knockout of *mbnT* via double homologous recombination was confirmed by screening the kanamycin-sensitive and sucrose-resistant phenotype, by PCR, and by sequencing.

RNA extraction and RT. RNA was isolated using a method described previously (23). Briefly, 2.5 ml of stop solution (5% buffer equilibrated phenol [pH 7.3] in ethanol) was first added to cultures (22.5 ml) to stop synthesis of new mRNA. Cell pellets were then collected by centrifugation at 4,300 × *g* for 15 min at 4°C. The cells were resuspended in 0.75 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 1.5 M NaCl, and 1% [wt/

TABLE 1 Primers used in this study

Primer	Targeted gene	Sequence[a] (5′–3′)	Reference
Arm A forward	*mbnT*	ATTTTTgaattcCCAGAAATATGAGATTCCGC[b]	This study
Arm A reverse		ATTTTTggatccCACGACCAGATCGATGATAC[b]	
Arm B forward	*mbnT*	ATTTTTggatccTTCGGTTCGATCAACGAGG[b]	This study
Arm B reverse		ATTTTTaagcttGCCAATCAGCGTGGAGAACC[b]	
qpmoA_FO	*pmoA*	TTCTGGGGCTGGACCTAYTTC	48
qpmoA_RO		CCGACAGCAGCAGGATGATG	
qmmoX_FO	*mmoX*	TCAACACCGATCTSAACAACG	48
qmmoX_RO		TCCAGATTCCRCCCCAATCC	
q16S rRNA_FO	16S rRNA	GCAGAACCTTACCAGCTTTTGAC	48
q16S rRNA_RO		CCCTTGCGGGAAGGAAGTC	
qmbnA_FO	*mbnA*	TGGAAACTCCCTTAGGAGGAA	23
qmbnA_RO		CTGCACGGATAGCACGAAC	

[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, BamHI, or HindIII restriction site sequences included in these primers.

vol] hexadecyltrimethylammonium bromide [CTAB]) before lysis using 20% SDS, 20% lauryl sarcosine, and bead beating. Subsequent steps of RNA extraction were then performed as described previously (23–25). Total RNA was then subjected to RNase-Free DNase treatment until free of DNA contamination as proven via PCR amplification of the 16S rRNA gene. The purified RNA was quantified spectrophotometrically using NanoDrop (NanoDrop ND-1000; NanoDrop Technologies, Inc., Wilmington, DE). RNA samples were stored at −80°C and were used for cDNA synthesis within 2 days of extraction. DNA-free total RNA (500 ng) was treated with SuperScript III reverse transcriptase for reverse transcription (RT) of mRNA to cDNA (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions.

RT-qPCR. Reverse transcription-quantitative PCR (RT-qPCR) analyses were performed to determine the relative expression of the *pmoA*, *mmoX*, and *mbnA* genes in *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant strains grown at various concentrations of copper and methanobactin. Gene-specific primers (Table 1) were used for the RT-qPCR analyses, and their specificity was verified by sequencing and gel electrophoresis. Measurements were performed in 96-well PCR plates using the CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA). In each well, quantitative PCRs (qPCRs) (20 μl) consisted of 0.8 μl cDNA, 1× iTaq universal SYBR green supermix (Bio-Rad, Hercules, CA), 0.5 μM each of the forward and reverse primers, and nuclease-free sterile water (Ambion/Life Technologies, Grand Island, NY). A three-step thermal cycler program, with an initial denaturation at 95°C for 3 min and 40 cycles of denaturation (94°C for 20 s), annealing (58°C for 20 s), and extension (68°C for 30 s), was performed. The specificity of qPCR products was again confirmed by melting curve analysis with temperatures ranging from 55°C to 95°C after the completion of amplification cycles. The threshold amplification cycle (C_T) values were then imported from CFX Manager software (Bio-Rad) into Microsoft Excel to quantify the relative levels of expression of different genes. The comparative C_T method ($2^{-\Delta\Delta CT}$) (28) was used to calculate relative gene expression levels using 16S rRNA as the housekeeping gene.

Metal analysis. Copper associated with the biomass of wild-type *M. trichosporium* OB3b and the *mbnT*::Gm^r mutant was determined as described previously (29). Briefly, cultures were harvested by centrifugation at 4,300 × *g* for 15 min. The cell pellets were resuspended in 1 ml morpholinepropanesulfonic acid (MOPS) buffer before being stored at −80°C. Before metal measurement, 1 ml of 70% nitric acid (vol/vol) was added to the cell suspension and was incubated for 2 h at 95°C with inversion every 20 min. Copper associated with biomass was subsequently analyzed using an inductively coupled plasma mass spectrometer (Agilent Technologies, Santa Clara, CA). At least duplicate biological samples for every condition were analyzed.

Methanobactin in spent medium and in cell extracts. For characterization of the location of methanobactin from wild-type *M. trichosporium* OB3b and the *mbnT*::Gm^r mutant, cells were cultured in 12 liters of NMS medium amended with 0.2 μM $CuCl_2$ in a 15-liter New Brunswick fermentor at 30°C for 48 h. Following the incubation period, 10 liters of the culture was removed and 10 liters of fresh NMS medium was added to the fermentor, and the copper concentration increased to 5 μM. This sequence was then repeated with increasing copper concentrations to 10 and 20 μM in subsequent fermentor turnovers.

The extracellular fraction and cells from each 10-liter sample were separated via tangential-flow filtration using a 10,000-Da molecular mass filter as previously described (30). The cells from the retentate were then harvested by centrifugation at 13,200 × *g* at 4°C. The pellet was resuspended in 10 mM phosphate buffer, pH 7.3, and was centrifuged at 13,200 × *g* at 4°C. This cell pellet was then resuspended in a minimal volume of 10 mM phosphate buffer at a pH of 6.8 plus 1 μg DNase · ml^{-1} and was lysed by three passes through an EmulsiFlex-C3 high-pressure homogenizer at 15,000 lb/in^2 (Avestin Inc., Ottawa, ON, Canada) at 4°C. The cell extract was then centrifuged at 13,000 × *g* for 20 min to remove unlysed cells followed by filtration though 0.2-μm Millipore filters (Billerica, MA).

Methanobactin antibody generation. Antibodies to methanobactin (Amb) from *M. trichosporium* OB3b were produced in LOU/c rats, which were immunized subcutaneously and intraperitoneally with a methanobactin-ovalbumin fusion protein (50 μg), 5 nmol CpG oligonucleotide (Tib Molbiol, Berlin, Germany), 500 μl phosphate-buffered saline, and 500 μl incomplete Freund's adjuvant. A boost without adjuvant was given 6 weeks after the primary injection. Tissue culture supernatants (TCS) were tested in a solid-phase immunoassay with methanobactin coupled to bovine serum albumin (BSA) or an irrelevant peptide coupled to BSA-coated enzyme-linked immunosorbent assay (ELISA) plates at a concentration of 4 μg · ml^{-1}. Monoclonal antibodies (MAbs) from TCS bound to methanobactin were detected with horseradish peroxidase (HRP)-conjugated MAbs against the rat IgG isotypes (TIB173 IgG2a, TIB174 IgG2b, and TIB170 IgG1 [all from the ATCC] and R-2c IgG2c [homemade]), thus avoiding MAbs of the IgM class. HRP was visualized with ready-to-use 3,3′,5,5′-tetramethylbenzidine (TMB) (1-step Ultra TMB ELISA; Thermo Fisher, Waltham, MA). Hybridomas that reacted specifically with methanobactin were frozen, and the antibody containing TCS was used in subsequent blots.

Derivation of polyvinylidene difluoride membranes. The N terminus of methanobactin from *M. trichosporium* OB3b is lost during ring formation (19, 21), preventing methanobactin from binding to polyvinylidene difluoride (PVDF) membranes (31). Poly(allylamine) was therefore attached to PVDF membranes, which enables methanobactin binding via its C terminus. Poly(allylamine) was attached to PVDF membranes by the derivatization procedure described by Rodrigues et al. (32). Briefly, PVDF sheets were etched in alcoholic KOH and then reacted with poly(allylamine) under alkaline conditions. Next, the amino groups were reacted with 1,4-phenylene diisothiocyanate (DITC), converting the amino-modified PVDF to DITC-functionalized membranes (DITC-phosphonoacetic acid [PAA]-PVDF membranes).

Chemiluminescence Western dot blots. DITC-PAA-PVDF transfer membranes were sized to fit an 8-by-12 well Bio-Dot dot blot (Bio-Rad Inc., Hercules, CA); they were washed with 20 mM Tris-HCl plus 0.5 M NaCl (Tris-buffered saline [TBS]) at pH 7.5 and were loaded with filter paper onto this dot blotter. Samples (spent medium and cell extracts) were then loaded under vacuum and were dried for 30 min under vacuum. The membrane was then wetted with 50% CH_3OH-50% H_2O and was washed twice in TBS at room temperature. The membrane was then incubated overnight in 0.2% nonfat dry milk in TBS at 4°C with the TBS subsequently decanted. The membrane was then resuspended in TBS plus 0.1% Tween 20 (TTBS) at pH 7.5 and incubated for 10 min at room temperature. The membrane was then suspended in fresh TTBS and incubated at room temperature for 1 h. The TTBS was decanted, and the membrane was resuspended in the primary antibody buffer (TTBS plus 0.2% nonfat dry milk [antibody buffer] plus 5.7 μg Amb · ml^{-1} [primary antibody solution]) and incubated overnight at 4°C. Following incubation, the primary antibody solution was decanted, and the membrane was resuspended in TTBS and incubated for 10 min with agitation, followed by one change in TTBS with incubation for an additional 10 min. The TTBS was decanted, and the membrane was resuspended in secondary antibody solution consisting of 33 μl of goat anti-rat Ig (H/L)-alkaline phosphatase from AbD Serotec (Atlanta, GA) to 100 ml of antibody buffer and incubated for 2 h. Following incubation, the secondary antibody solution was decanted, and the membrane was washed three times with TTBS as described above. Visualization of the blot was done via the Bio-Rad Immun-Star AP substrate for chemiluminescence (Hercules, CA, USA) by following the manufacturer's suggested procedure.

RESULTS

Using marker exchange protocols, a transconjugant colony with a double homologous recombination event in which *mbnT* was suc-

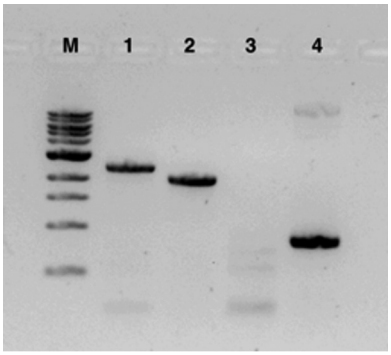

FIG 2 Verification of knockout of *mbnT* in *M. trichosporium* by PCR. M, molecular weight markers; lane 1, PCR of *mbnT* from the *M. trichosporium* OB3b *mbnT*::Gm^r mutant; lane 2, PCR of *mbnT* from wild-type *M. trichosporium* OB3b; lane 3, PCR of pK18*mobsacB* backbone in *M. trichosporium* OB3b *mbnT*::Gm^r; lane 4, PCR of pK18*mobsacB* backbone in pWG011.

cessfully knocked out was identified (Fig. 2). This was confirmed by sequencing as well as by verifying that the mutant was gentamicin and sucrose resistant but sensitive to kanamycin (data not shown).

The phenotype of the *mbnT*::Gm^r mutant was then further examined and compared to that of wild-type *M. trichosporium*. When grown in various copper concentrations, both the *mbnT*::Gm^r mutant and the wild type had increasing amounts of copper associated with biomass (Fig. 3A). Further, gene expression in both the mutant and the wild type showed clear evidence of the copper switch; i.e., as copper increased, expression of *mmoX* decreased by several orders of magnitude, while *pmoA* expression increased over an order of magnitude (Fig. 3B and C). Finally, expression of *mbnA*, encoding the precursor polypeptide of methanobactin, decreased substantially in wild-type *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant as copper increased, indicating that the knocking out of *mbnT* did not affect methanobactin expression (Fig. 3D).

These findings suggest either that *mbnT* is not involved in copper uptake (i.e., binding of copper-methanobactin complexes) or that there are multiple mechanisms for copper uptake in *M. trichosporium* OB3b, such that the copper switch is still operative. To differentiate between these possibilities, methanobactin in the spent medium and cell extracts of the *mbnT*::Gm^r mutant and wild-type strain of *M. trichosporium* OB3b was assayed for a wide range of copper concentrations using immunoblotting assays. As shown in Fig. 4, as the growth concentration of copper increased, the amount of methanobactin in the spent medium decreased in wild-type *M. trichosporium* OB3b but was readily apparent in the spent medium of the *mbnT*::Gm^r mutant at all tested copper concentrations. Conversely, methanobactin was found in the cell extract of *M. trichosporium* OB3b under all conditions, indicating that methanobactin was taken up after secretion. No methanobactin was ever observed in the cell extract of the *mbnT*::Gm^r mutant, indicating that the mutant produced and secreted methanobactin but was unable to subsequently take it up.

The *mbnT*::Gm^r mutant and wild-type strain of *M. trichosporium* OB3b were then grown in the presence of 1 μM copper and various amounts of copper-free methanobactin. As shown in Fig. 5A, in the presence of either 5 or 50 μM methanobactin, copper associated with the biomass of the *mbnT*::Gm^r mutant decreased

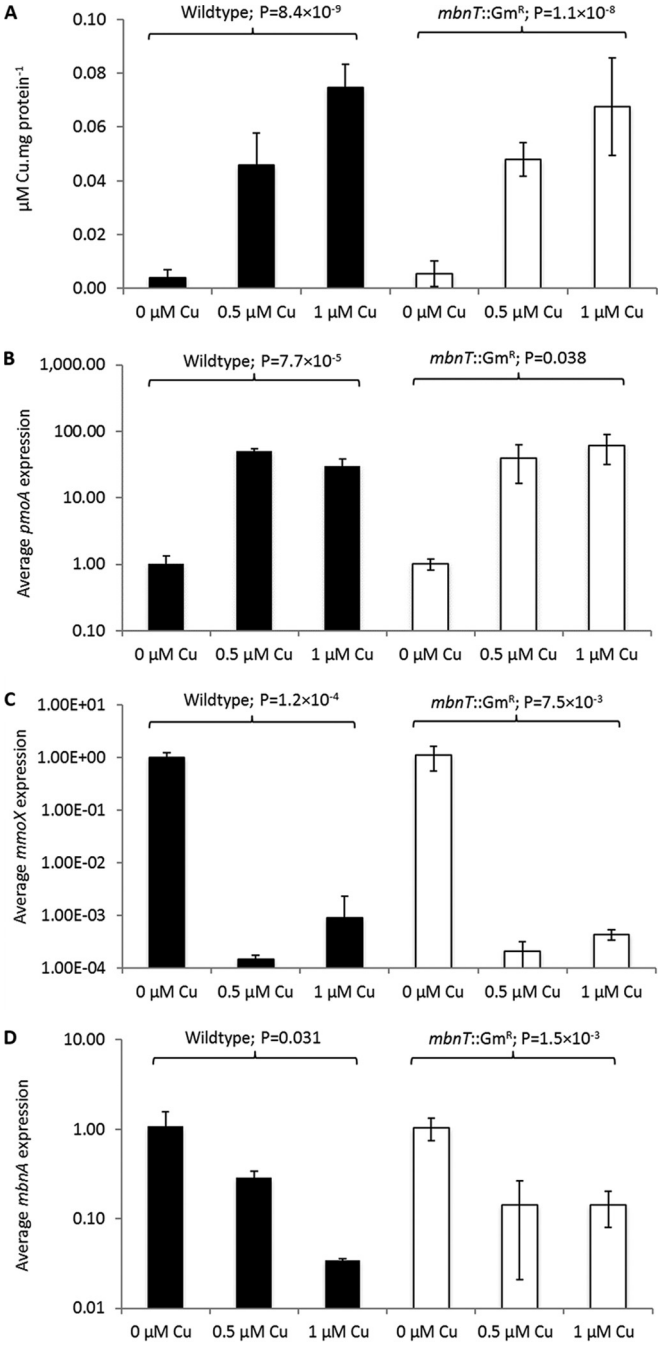

FIG 3 Characterization of wild-type *M. trichosporium* OB3b (black bars) and the *mbnT*::Gm^r mutant (white bars) grown in the presence of various amounts of copper. (A) Copper associated with biomass; (B) RT-qPCR of *pmoA*; (C) RT-qPCR of *mmoX*; (D) RT-qPCR of *mbnA*. Error bars indicate standard deviations from at least duplicate biological replicates. Indicated *P* values are from one-way analysis of variance (ANOVA).

>3-fold, while no significant change in the copper levels of wild-type *M. trichosporium* OB3b was observed. Further, expression of *mmoX* increased >3 orders of magnitude in the *mbnT*::Gm^r mutant, while *pmoA* expression dropped by approximately 8-fold. No significant change in the expression of either *mmoX* or *pmoA* was observed, however, in wild-type *M. trichosporium* OB3b

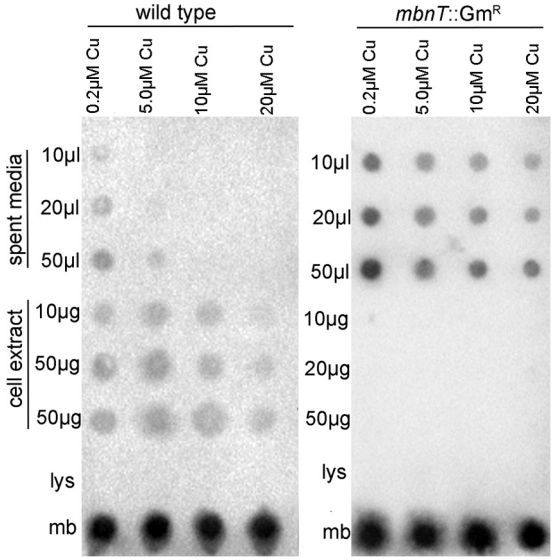

FIG 4 Immuno-blotting assays for location of methanobactin in wild-type *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant as a function of the concentration of copper in the growth medium (0.2, 5, 10, or 20 μM copper). Fifty nanomoles lysozyme (lys) and 50 nmol methanobactin (mb) were used as negative and positive controls, respectively.

(Fig. 5B and C). Collectively, these data show that in the presence of a molar excess of methanobactin, copper was still bioavailable to wild-type *M. trichosporium* OB3b but was not for the *mbnT*::Gm^r mutant. Additionally, it was assayed whether the addition of exogenous methanobactin affected *mbnA* expression in wild-type *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant. As shown in Fig. 5D, as increasing amounts of methanobactin were added, *mbnA* expression increased in both the wild-type and mutant strains.

DISCUSSION

Since the discovery of the methanobactin gene cluster, it has been speculated that a TonB-dependent transporter encoded by *mbnT* is responsible for methanobactin uptake (23). Here, we show that methanobactin uptake is indeed mediated by *mbnT*, as (i) methanobactin was taken up by wild-type *M. trichosporium* OB3b but not by the *mbnT*::Gm^r mutant and (ii) the *mbnT*::Gm^r mutant of *M. trichosporium* OB3b was unable to take up copper if methanobactin was exogenously added to bind copper, but wild-type *M. trichosporium* OB3b was able to take up copper.

The data also show, however, that *M. trichosporium* OB3b has an alternative mechanism(s) for copper uptake; i.e., in the absence of any exogenous methanobactin, the amounts of copper in the wild-type and *mbnT*::Gm^r strains of *M. trichosporium* OB3b were indistinguishable. The conclusion of multiple copper uptake systems, however, is not novel, as it was reported earlier that at least two pathways for copper uptake exist in *M. trichosporium* OB3b (33). Such redundancy in copper uptake systems in methanotrophs, although unusual compared to those of other microbes, can be explained when one considers the importance of copper in methanotrophic metabolism. That is, methanotrophs expressing pMMO have a strong need for copper, as it occupies at least two of three metal centers found in purified pMMO (4, 33–35).

An interesting issue is that, as found earlier in a mutant of *M. trichosporium* OB3b where *mbnA*, encoding the precursor polypeptide of methanobactin, was knocked out, the copper switch still existed in the *mbnT*::Gm^r mutant. Genomic analyses have found that *mbnT* is part of a FecIRA-like gene cluster; i.e., *mbnT* is

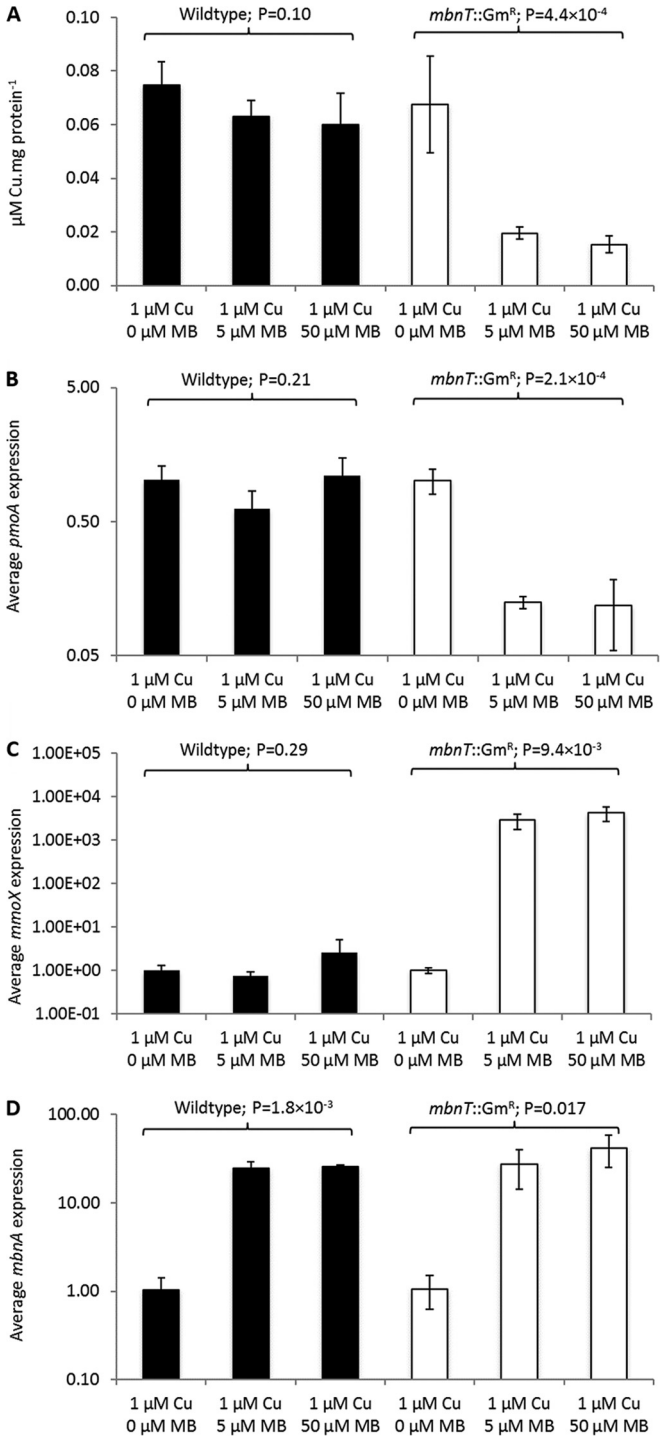

FIG 5 Characterization of wild-type *M. trichosporium* OB3b (black bars) and the *mbnT*::Gm^r mutant (white bars) grown in the presence of 1 μM copper and various amounts of methanobactin (MB). (A) Copper associated with biomass; (B) RT-qPCR of *pmoA*; (C) RT-qPCR of *mmoX*; (D) RT-qPCR of *mbnA*. Error bars indicate standard deviations from at least duplicate biological replicates. Indicated *P* values are from one-way analysis of variance (ANOVA).

preceded by *mbnR* and *mbnI*, encoding a putative membrane sensor and an extracytoplasmic function sigma factor, respectively (36). Such a system is frequently found in siderophore synthesis where an outer membrane transporter binds a ferrisiderophore, transmitting a signal to a membrane sensor that then activates an extracytoplasmic function sigma factor. This ultimately induces the expression of genes required for siderophore synthesis, as well as, in some cases, genes unrelated to siderophore production or uptake, e.g., genes encoding exotoxins and proteases (37–43). Given this similarity, it has been speculated that after MbnT binds copper-methanobactin, a signal cascade results whereby methanobactin synthesis and possibly expression of *mmo* and *pmo* operons are controlled (36).

The findings presented here, however, suggest that although such a signal cascade may exist after MbnT binds copper-methanobactin, such a regulatory scheme does not include the copper switch between sMMO and pMMO. It is also difficult to conclude from our data that this signal cascade affects the expression of *mbnA*. That is, *mbnA* expression in wild-type *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant decreased significantly with increasing copper, but the magnitude of the drop in expression was greater in the wild-type strain (Fig. 3D). Further, in the presence of 1 μM copper and various amounts of exogenous methanobactin, *mbnA* expression in wild-type *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant responded with the same pattern (Fig. 5D). It appears that another regulatory circuit is involved in controlling the expression of *mbnA*, but the possibility that such expression is also controlled to some extent by *mbnI*, which is indirectly activated by MbnT binding copper-methanobactin, cannot be excluded at this time.

In conclusion, here we report the successful knockout of *mbnT* and show that this is responsible for methanobactin uptake. The phenotype of the *mbnT*::Gm^r mutant, however, indicates that *M. trichosporium* OB3b has multiple systems for copper uptake. It is tempting to speculate that methanobactin may serve as a high-affinity system to collect copper, but when copper is not limiting, an alternative lower-affinity system is used. Such a hypothesis is supported by the finding that expression of *mbnA* decreases with increasing copper in wild-type *M. trichosporium* OB3b and in the *mbnT*::Gm^r mutant.

The nature of this imputed low-affinity copper uptake mechanism is still elusive, but clues from other methanotrophs, e.g., *Methylomicrobium album* BG8 and *Methylococcus capsulatus* Bath, may provide some suggestions. That is, it has been shown that in *M. album* BG8, there exists an outer membrane protein, CorA, that is copper repressible and may serve to bind copper (44). Further, it has been found that *M. capsulatus* Bath synthesizes a similar outer membrane protein, MopE, as well as a secreted truncated form, MopE*, both of which bind Cu(II) (45–47). A gene encoding a protein similar to CorA and MopE, *mbnP*, is adjacent to the methanobactin gene cluster in *M. trichosporium* OB3b (36), and it may be that this serves as an alternative copper uptake mechanism in *M. trichosporium* OB3b. To determine if this is indeed the case, it is recommended that the protein and lipid composition of the outer membrane of *M. trichosporium* OB3b be characterized under various copper concentrations to see if any significant changes in MbnP occur. It may also be informative to create double knockouts, e.g., knockouts of both *mbnP* and *mbnT* or *mbnP* and *mbnA*, to determine if the resulting double mutants of *M. trichosporium* OB3b are severely inhibited in their ability to collect copper.

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