

## ARTICLE



# Evidence for methanobactin “Theft” and novel chalkophore production in methanotrophs: impact on methanotrophic-mediated methylmercury degradation

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Aerobic methanotrophy is strongly controlled by copper, and methanotrophs are known to use different mechanisms for copper uptake. Some methanotrophs secrete a modified polypeptide—methanobactin—while others utilize a surface-bound protein (MopE) and a secreted form of it (MopE\*) for copper collection. As different methanotrophs have different means of sequestering copper, competition for copper significantly impacts methanotrophic activity. Herein, we show that *Methylobacterium album* BG8, *Methylocystis* sp. strain Rockwell, and *Methylococcus capsulatus* Bath, all lacking genes for methanobactin biosynthesis, are not limited for copper by multiple forms of methanobactin. Interestingly, *Mm. album* BG8 and *Methylocystis* sp. strain Rockwell were found to have genes similar to *mbnT* that encodes for a TonB-dependent transporter required for methanobactin uptake. Data indicate that these methanotrophs “steal” methanobactin and such “theft” enhances the ability of these strains to degrade methylmercury, a potent neurotoxin. Further, when *mbnT* was deleted in *Mm. album* BG8, methylmercury degradation in the presence of methanobactin was indistinguishable from when MB was not added. *Mc. capsulatus* Bath lacks anything similar to *mbnT* and was unable to degrade methylmercury either in the presence or absence of methanobactin. Rather, *Mc. capsulatus* Bath appears to rely on MopE/MopE\* for copper collection. Finally, not only does *Mm. album* BG8 steal methanobactin, it synthesizes a novel chalkophore, suggesting that some methanotrophs utilize both competition and cheating strategies for copper collection. Through a better understanding of these strategies, methanotrophic communities may be more effectively manipulated to reduce methane emissions and also enhance mercury detoxification in situ.

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## INTRODUCTION

Aerobic methanotrophs play a critical role in the biogeochemical cycling of carbon [1, 2]. More specifically, these intriguing microbes consume substantial amounts of methane generated via methanogenesis [3], and thus may be invaluable means or “levers” to control not only future emissions of methane, but also remove methane from the atmosphere [4–8].

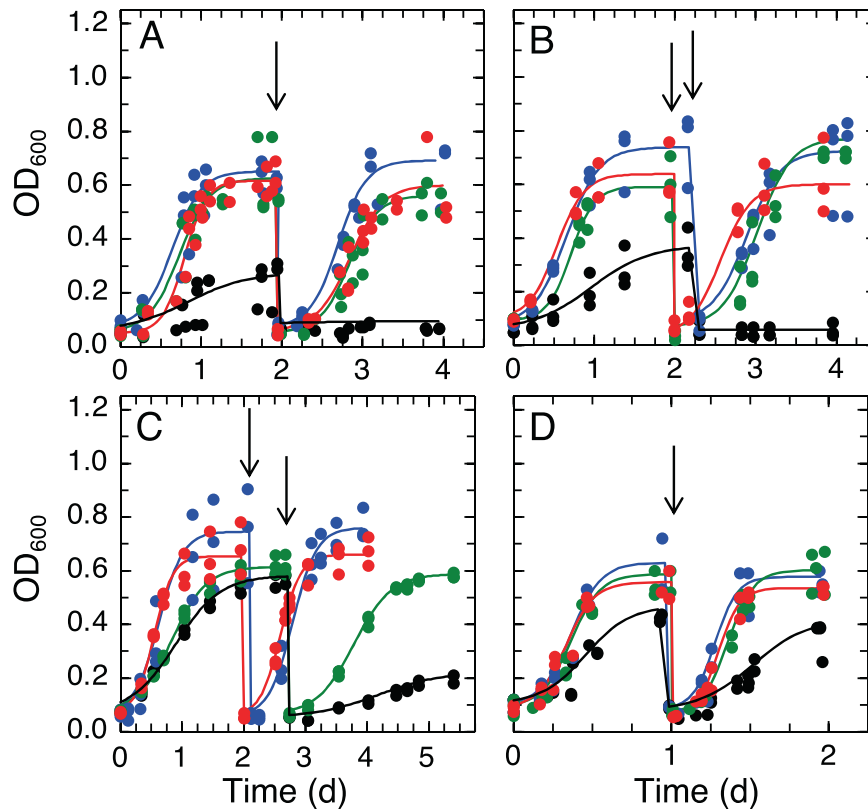
Any application of aerobic methanotrophy, however, requires a detailed understanding of their metabolism, particularly their need for trace metals. Specifically, expression and activity of alternative forms of methane monooxygenase (MMO, responsible for the conversion of methane to methanol), are controlled by copper, or the canonical “copper-switch” [9]. There are two forms of MMO—a cytoplasmic or soluble methane monooxygenase (sMMO) and a membrane-bound or particulate methane monooxygenase (pMMO). sMMO—a soluble di-iron-containing enzyme

[10]—is only expressed under copper limitation [11, 12]. Expression and activity of pMMO—a copper and iron-containing enzyme [13, 14]—increases with increasing copper [11, 12]. The two forms of MMO have widely different properties, e.g., cells expressing sMMO have high methane turnover but poor affinity for methane while pMMO-expressing cells have lower turnover but greater affinity [15]. Given that methane oxidation is critical for methanotrophic growth, copper sequestration is also very important to these microbes, and methanotrophs have been found to have multiple mechanisms of copper uptake.

The first well-characterized copper-binding compound or chalkophore—methanobactin (MB)—is secreted by some methanotrophs of the *Methylocystaceae* family within the Alphaproteobacteria, e.g., *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain SB2 [16–18]. MB is a modified polypeptide containing two heterocyclic rings with associated thioamide groups that are

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**Fig. 1** Methanotrophic growth in the presence of varying amounts of copper and methanobactin. Growth of **A** *Mm. album* BG8 wild type, **B** *Mm. album* BG8  $\Delta mbnT$ , **C** *Methylocystis* sp. strain Rockwell, and **D** *Mc. capsulatus* Bath with 0  $\mu\text{M}$  Cu (black), 1  $\mu\text{M}$  Cu (blue), 1  $\mu\text{M}$  Cu, and 5  $\mu\text{M}$  OB3b-MB (green), and 1  $\mu\text{M}$  Cu and 5  $\mu\text{M}$  SB2-MB (red). Solid lines indicate data fitted to a logistic growth curve using growthcurver [48], and arrows indicate the beginning of second growth cycle.

responsible for copper binding with extremely high affinity [19]. To date, two forms of MB have been described—Group I MBs with two oxazolone rings and an internal disulfide bridge (e.g., MB from *Ms. trichosporium* OB3b) and Group II MBs that contain one oxazolone ring and one pyrazinedione or imidazolone ring as well as a sulfate group (e.g., MB from *Methylocystis* sp. strain SB2 [17, 19]). The gene encoding the polypeptide precursor of MB has been identified (*mbnA* [11]) as have several genes involved in ring formation (*mbnBCN* [20, 21]) and MB uptake (*mbnT*, encoding a TonB-dependent transporter [22]). Not all methanotrophs, however, can produce MB. Rather, methanotrophs of the *Methylococcaceae* family of the Gammaproteobacteria rely on an outer membrane protein (MopE) and a secreted form of this molecule (MopE\*) for copper sequestration [23–27]. Finally, some *Methylocystaceae* methanotrophs lack both MB and the MopE/MopE\* systems for copper uptake, suggesting that they collect copper by some unknown system(s) [28].

Given the importance of copper in methanotrophy, this raises several intriguing questions. First, do methanotrophs that express MB have a competitive advantage for copper sequestration? Competition between methanotrophs for copper is likely, with such competition affecting overall methanotrophic activity. Second, given that MB is secreted into the environment to collect copper, can copper-MB complexes be “stolen” by other microbes? Such a phenomenon would require non-MB-expressing methanotrophs to have the uptake system identified for MB, i.e., MbnT [22, 29]. Such “theft” would not be unprecedented, as many microbes have been found to act as “cheaters” where they steal siderophores produced by others to collect iron [30–33]. Further, it has been found that methanotrophs that produce and take up MB are able to degrade the potent neurotoxin methylmercury (MeHg) [34]. If some methanotrophs act as MB-cheaters, does such theft

enable these microbes to degrade MeHg? Herein we examine several methanotrophs unable to produce MB to determine: (1) if copper requirements of these methanotrophs can be met either through MB theft and/or competition (i.e., expression of some other copper uptake system) and (2) if MB theft enables these microbes to degrade MeHg.

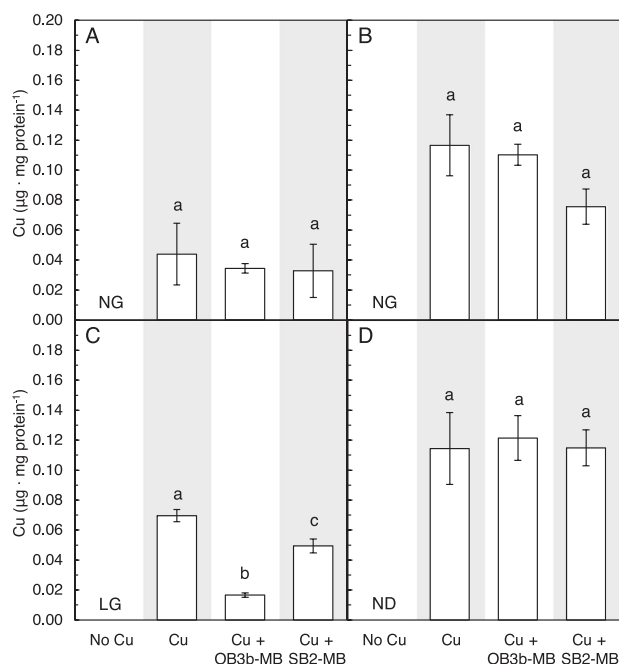
## MATERIALS AND METHODS

### Identification of putative MbnTs in *Mm. album* BG8, *Methylocystis* sp. strain Rockwell, and *Mc. capsulatus* Bath

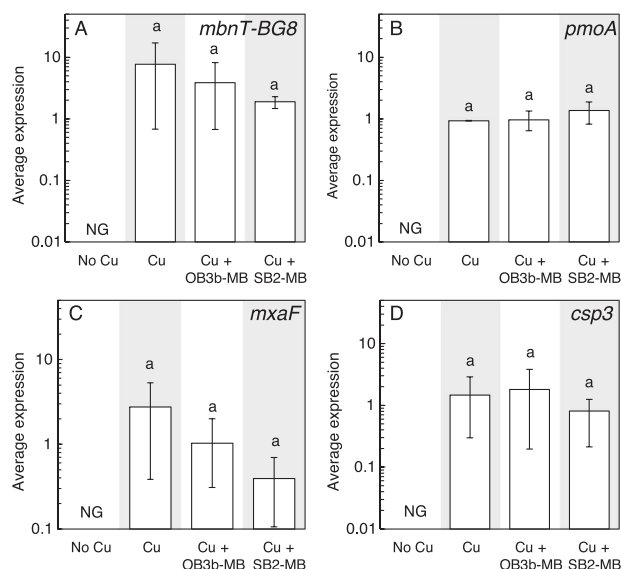
Predicted MbnT amino acid sequences of *Ms. trichosporium* OB3b (ADVE02\_v2\_13651) and *Methylocystis* sp. strain SB2 (MSB2v1\_460017) were used to search for putative *mbnT* genes in the genomes of *Mm. album* BG8, *Methylocystis* sp. strain Rockwell, and *Mc. capsulatus* Bath using tblastn or blastp ([35]).

### Growth conditions

Initial inocula of *Mm. album* BG8 and *Mc. capsulatus* Bath were grown in nitrate mineral salts (NMS) while *Methylocystis* sp. strain Rockwell was grown in ammonium mineral salts (AMS) media [36], all with 1  $\mu\text{M}$  copper (as  $\text{CuCl}_2$ ). *Mm. album* BG8 and *Methylocystis* sp. strain Rockwell were grown at 30 °C, and *Mc. capsulatus* Bath at 45 °C in 250-mL sidearm Erlenmeyer flasks while shaken at 220 rpm in the dark.  $\text{CH}_4$  was supplemented at a  $\text{CH}_4$ -to-air ratio of 1:2. MBs from *Ms. trichosporium* OB3b (OB3b-MB) and *Methylocystis* sp. strain SB2 (SB2-MB) were purified as described previously [37]. Cu-MB stocks were freshly prepared by adding  $\text{CuCl}_2$  and either OB3b-MB or SB2-MB at 1:5 molar ratio and incubating in the dark at 30 °C for 1 h [38]. Cu-triethylenetetramine (TRIEN, a strong abiotic chelator of copper [39]) was prepared by adding  $\text{CuCl}_2$  and TRIEN at 1:5 molar ratio. To investigate the effect of copper chelation on the methanotrophic growth, cultures grown with 1  $\mu\text{M}$  copper were washed with fresh NMS or AMS media and then transferred to four different conditions: 0  $\mu\text{M}$  Cu, 1  $\mu\text{M}$  Cu, 1  $\mu\text{M}$  Cu + 5  $\mu\text{M}$  OB3b-MB, and 1  $\mu\text{M}$  Cu + 5



**Fig. 2 Copper associated with methanotrophic biomass in the presence of varying amounts of copper and methanobactin.** Copper associated with **A** *Mm. album* BG8 wild type, **B** *Mm. album* BG8  $\Delta mbnT$ , **C** *Methylocystis* sp. strain Rockwell, and **D** *Mc. capsulatus* Bath biomass at the end of the second growth cycle with 0  $\mu\text{M}$  Cu, 1  $\mu\text{M}$  Cu, and 5  $\mu\text{M}$  OB3b-MB, 1  $\mu\text{M}$  Cu and 5  $\mu\text{M}$  SB2-MB. Error bar indicates the standard deviation of biological triplicate samples. Letter over bars indicates no significant differences determined by Tukey's honestly significant difference test ( $p < 0.05$ ). No detected (ND) copper associated with biomass and no growth (NG) is indicated. Low growth (LG) indicates insufficient biomass for metal analysis.



**Fig. 3 Gene expression in *Mm. album* BG8 wild type.** RT-qPCR of **A** *mbnT*-BG8, **B** *pmoA*, **C** *mxoF*, and **D** *csp3* in *Mm. album* BG8 wild type grown with or without 1  $\mu\text{M}$  Cu and 5  $\mu\text{M}$  MB. Error bar indicates the range of biological duplicate or triplicate samples. Letter over bars indicates no significant differences determined by Tukey's honestly significant difference test ( $p < 0.05$ ). No growth is indicated as NG.

$\mu\text{M}$  SB2-MB. *Mm. album* BG8 and *Methylocystis* sp. strain Rockwell were also grown in the presence of TRIEN to determine if these strains produce novel chalkophores in the presence of an abiotic competitive ligand. After reaching the stationary phase, all cultures were washed with fresh NMS or AMS media and used to inoculate a new set of flasks for a second growth cycle. Methanotrophic growth in the presence of varying amounts of copper and copper-chelating agents was non-invasively monitored by measuring the optical density at 600 nm ( $\text{OD}_{600}$ ) in sidarm flasks using a Genesys 20 Visible spectrophotometer (Spectronic Unicam, Waltham, MA). All conditions were run in biological triplicates.

*Escherichia coli* used for mutant construction in *Mm. album* BG8 was grown in Luria broth medium (Dot Scientific, Burton, MI). Kanamycin was used for maintaining *E. coli* and *Mm. album* BG8 containing mutant construct plasmids (25 and 10  $\mu\text{g mL}^{-1}$ , respectively). Nalidixic acid (15  $\mu\text{g mL}^{-1}$ ) was used to remove residual *E. coli* strain S17-1 after conjugation with *Mm. album* BG8.

### RNA extraction and cDNA synthesis

Total RNA from cultures at the end of the second growth cycle was extracted as described previously [11, 40]. Removal of DNA was confirmed by the absence of products from polymerase chain reaction (PCR) with the universal primers 27F and 1492R targeting 16S rRNA gene (Table S1). cDNA was synthesized from DNA-free RNA samples using Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Genes involved in methane oxidation (*pmoA* and *mmoX*), methanol oxidation (*mxoF*), putative MB uptake (Metal\_1282 in *Mm. album* BG8, hereafter called *mbnT*-BG8; MSPATv1\_230027 and MSPATv1\_550006 in *Methylocystis* sp. strain SB2, hereafter called *mbnT1*-Rockwell and *mbnT2*-Rockwell; MCA1957 in *Mc. capsulatus* Bath, hereafter called *mbnT*-Bath; and *mopE* in *Mc. capsulatus* Bath (MCA2589)), and copper storage (*csp3* in *Mm. album* BG8 (Metal\_0689) and MSPATv1\_280020 in *Methylocystis* sp. strain Rockwell, hereafter called *csp1*) were quantified by RT-qPCR. Expression of 16S rRNA genes was used as an internal reference. Primer sets used for qPCR of these select genes are shown in Table S1 and calibration curves generated as shown in Figs. S1–S3. qPCR reactions were performed as described previously [40]. Threshold cycle ( $C_T$ ) values were imported from CFX Manager Software (Bio-Rad, Hercules, CA) to calculate relative gene expression levels with 16S rRNA as the internal standard by the comparative threshold amplification cycle method [41]. Measurements were performed for at least biological duplicates for each condition.

### Construction of *mbnT* mutant of *Mm. album* BG8

The gene most similar to any *mbnT* in *Mm. album* BG8 (Metal\_1282) was knocked out via markerless mutagenesis as described previously [20]. Primers used in this study are shown in Table S1. Deletion of the putative MB uptake gene in *Mm. album* BG8  $\Delta mbnT$  was confirmed by checking for kanamycin sensitivity as well as via PCR and sequencing (Fig. S4).

### Metal analysis

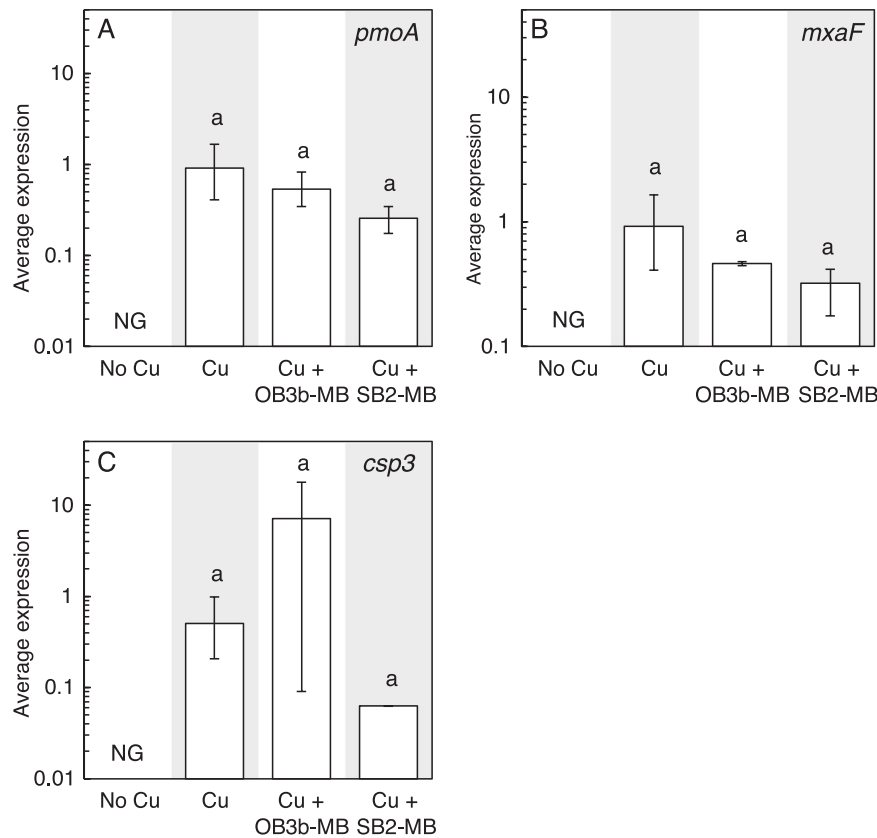
Copper uptake by *Mm. album* BG8 wild type and  $\Delta mbnT$  strains, *Methylocystis* sp. strain Rockwell, and *Mc. capsulatus* Bath at the end of the second growth cycle was determined as described previously [38]. Triplicate biological samples were analyzed for every condition.

### Immunoblotting

Monoclonal antibody (10B10) to MB from *Ms. trichosporium* OB3b was produced and purified as described earlier [22, 42]. 1,4-phenylene diisothiocyanate-derivatized polyvinylidene difluoride membranes were modified as previously described [43], with changes as detailed in the supplementary material. A detailed method of immunoblotting is provided in the supplementary material.

### *Mm. album* BG8 chalkophore purification and characterization

To isolate a putative novel chalkophore produced by *Mm. album* BG8, cultures grown in the presence of 1  $\mu\text{M}$  copper and 5  $\mu\text{M}$  TRIEN were first centrifuged at 4300  $\times$  g for 10 min. Supernatant was collected and filtered through a 0.2- $\mu\text{m}$  PES filter unit (Thermo Scientific, Waltham MA). A



**Fig. 4** Gene expression in *Mm. album* BG8  $\Delta mbnT$ . RT-qPCR of **A** *pmoA*, **B** *mxoF*, and **C** *csp3* in *Mm. album* BG8  $\Delta mbnT$  grown with or without 1  $\mu$ M Cu and 5  $\mu$ M MB. Error bar indicates the range of biological duplicate or triplicate samples. Letter over bars indicates no significant differences determined by Tukey's honestly significant difference test ( $p < 0.05$ ). No growth is indicated as NG.

reversed-phase  $C_{18}$  Sep-Pak cartridge (Waters Corp., Milford MA) was sequentially conditioned with 3 mL methanol, 3 mL 60% acetonitrile, 3 mL methanol, and 6 mL  $H_2O$ , and then loaded with the filtered spent medium. The chalkophore bound to the column was washed with 6 mL  $H_2O$ , then eluted with 60% acetonitrile until a yellow band was collected. The eluent was frozen at  $-80^\circ C$  and lyophilized for chalkophore concentration and removal of acetonitrile (FreeZone 6 Freeze Dry System, Labconco, Kansas City MO). For larger scale production and isolation of the chalkophore from *Mm. album* BG8, cells were cultured in a 15 L Solida fermenter (Solida Biotechnology, Munich Germany) using the culture conditions described above. The chalkophore was isolated from the spent media and purified as described for MB from *Methylocystis* sp. strain SB2 [44] except the chalkophore from *Mm. album* BG8 was eluted from the Targa C18 column in the 65-75% methanol:  $H_2O$  fraction. UV-visible spectroscopy was recorded on a Cary 50 (Agilent, Santa Clara, CA). Matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS) was performed on a Shimadzu AXIMA Confidence MALDI TOF Mass Spectrometer (Shimadzu Corp., Kyoto, Japan) using a mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (SuperDHB) in a 1:1 matrix to sample mixture. Electrospray ionization (ESI)MS/MS was performed on an Agilent LC using a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer with an HCD fragmentation cell and an Agilent 1260 Infinity Capillary Pump with an Agilent Zorbax SB-C18, 0.5 mm  $\times$  150 mm, 5 micron, part#5064-8256 using 0.1% formic acid/water and 0.1% formic acid/acetonitrile.

#### Methylmercury degradation assays

MeHg degradation by *Mm. album* BG8 wild type and  $\Delta mbnT$ , *Methylocystis* sp. strain Rockwell, and *Mc. capsulatus* Bath was measured in biological triplicate samples as described earlier [34, 45–47]. Experiments were performed in the absence/presence of MB from either *Ms. trichosporium* OB3b or *Methylocystis* sp. strain SB2. The final concentrations of cells, MeHg, and MB (if added) were  $10^8$  cells  $mL^{-1}$ , 5 nM, and 45  $\mu$ M, respectively [46].

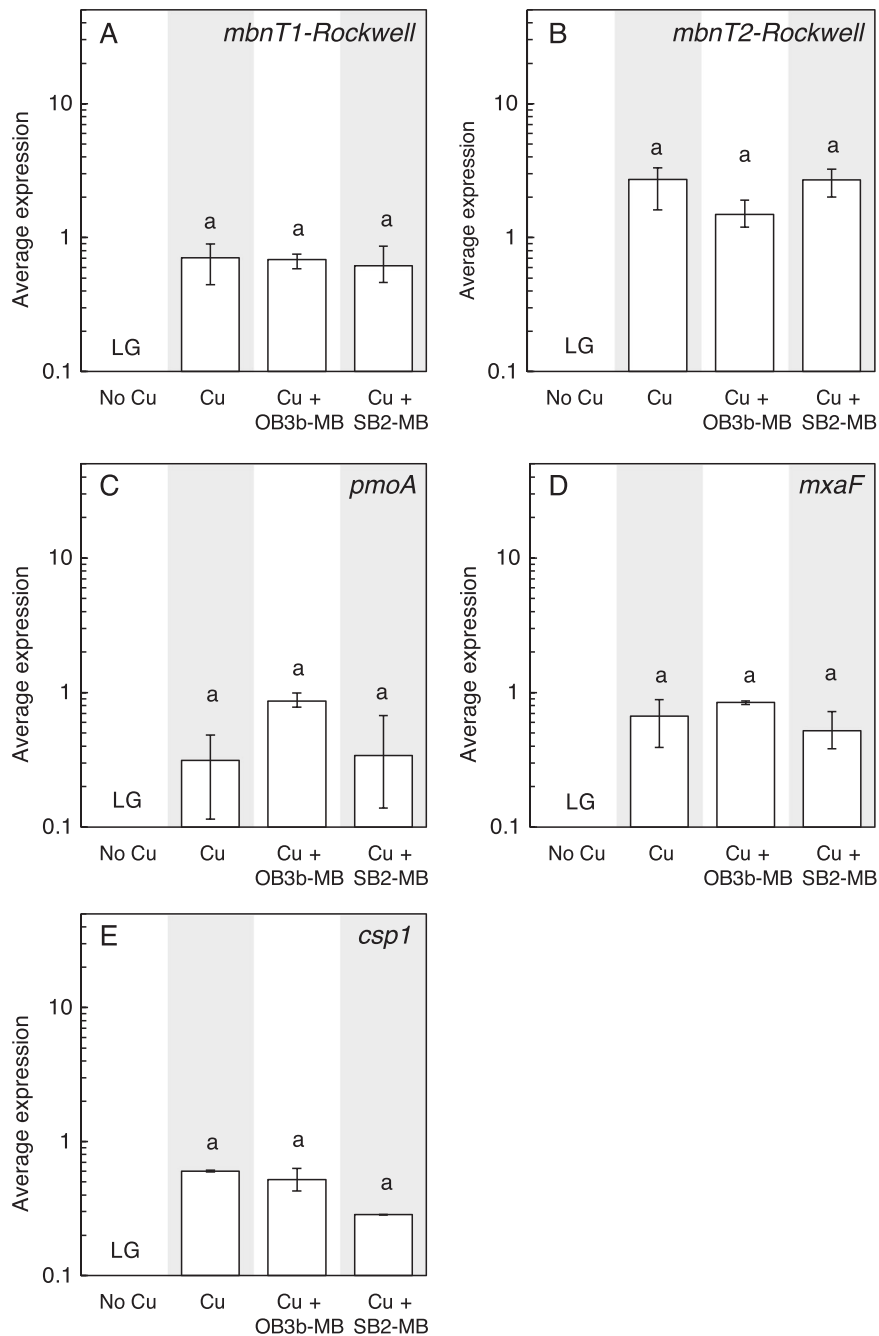
#### Statistical analyses

Data were analyzed using the Tukey's honestly significant difference test or Student's *t*-test. Potential outliers of biological triplicates were determined using the Grubbs' outlier test. Microbial growth was fitted to a logistic curve using the R package growthcurver [48] to calculate growth rates. All statistical analyses were performed using R version 3.4.4 [49].

## RESULTS

### Identification of putative MbnTs in *Mm. album* BG8, *Methylocystis* sp. strain Rockwell, and *Mc. capsulatus* Bath

In *Mm. album* BG8, one gene encoding for a putative TonB-dependent receptor was most similar to both OB3b-MbnT and SB2-MbnT (Metal\_1282; Table S2). Similarity was much greater, however, to OB3b-MbnT ( $E$  value =  $2 \times 10^{-145}$ , identity = 36%) than SB2-MbnT ( $E$  value =  $1 \times 10^{-12}$ , identity = 28%). For *Methylocystis* sp. strain Rockwell, several genes encoding for putative TonB-dependent transporters similar to OB3b-MbnT were found in the genome of *Methylocystis* sp. strain Rockwell, of which MSPATv1\_230027 exhibited the highest similarity ( $E$  value =  $1 \times 10^{-136}$ , identity = 36%; Table S2). Two genes encoding for putative TonB-dependent transporters highly similar to SB2-MbnT (MSPATv1\_550006 and MSPATv1\_50173;  $E$  value = 0.0 for both) were found in the genome of *Methylocystis* sp. strain Rockwell. The identification of MSPATv1\_550006 to SB2 MbnT, however, was much higher than that found for MSPATv1\_50173, i.e., 65% vs. 42%. For *Mc. capsulatus* Bath, no TonB-dependent transporter was found to have significant similarity to either OB3b-MbnT or SB2-MbnT, the closest being MCA1957 to OB3b-MbnT ( $E$  value =  $2 \times 10^{-6}$ , identity = 21%) and MCA2074 to SB2-MbnT ( $E$  value of  $9 \times 10^{-13}$ , identity = 28%). Expression of genes with high similarity to known *mbnT* genes, i.e., Metal\_1282 (*mbnT*-BG8),



**Fig. 5 Gene expression in *Methylocystis sp. Rockwell*.** RT-qPCR of **A** *mbnT1-Rockwell*, **B** *mbnT2-Rockwell*, **C** *pmoA*, **D** *mxoF*, and **E** *csp1* in *Methylocystis sp. Rockwell* grown with or without 1  $\mu$ M Cu and 5  $\mu$ M MB. Error bar indicates the range of biological duplicate or triplicate samples. Letter over bars indicates no significant differences determined by Tukey's honestly significant difference test ( $p < 0.05$ ). Low growth is indicated as LG.

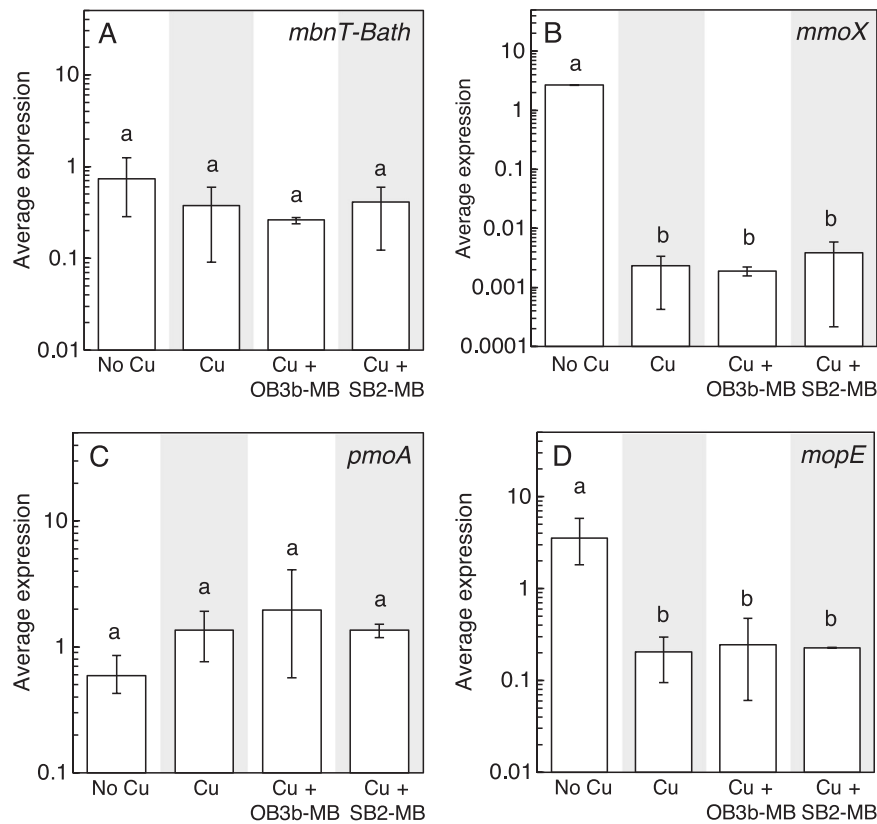
MSPATv1\_230027 (*mbnT1-Rockwell*), MSPATv1\_550006 (*mbnT2-Rockwell*), and MCA1957 (*mbnT-Bath*) was monitored under different growth conditions as described below.

#### Growth of *Mm. albus* BG8 wild type and $\Delta$ *mbnT* mutant in the presence of varying amounts of copper, MB, and TRIEN

Growth of *Mm. albus* BG8 wild type was strongly dependent on the availability of copper as described previously [50]. Growth clearly occurred in the presence of 1  $\mu$ M copper, but was significantly reduced with no added copper in the first growth cycle (final OD<sub>600</sub> of  $0.60 \pm 0.03$  vs.  $0.24 \pm 0.10$ ;  $p = 0.018$ ) (Fig. 1A, S5A). Growth was abolished when this culture was transferred a second time to copper-

free medium, indicating that original growth was likely due to the transfer of a small amount of copper with the initial inoculum. Such a result is not unexpected as *Mm. albus* BG8 can only express pMMO that requires copper for its activity.

The addition of either 5  $\mu$ M OB3b-MB (a Group I MB) or SB2-MB (a Group II MB) in the presence of 1  $\mu$ M copper did not affect the growth of *Mm. albus* BG8 as compared to growth in the presence of 1  $\mu$ M copper only, indicating that neither form of MB inhibited copper uptake (Fig. 1A, S5A). This was confirmed by measuring copper associated with biomass at the end of the second growth cycle—no significant difference was found for cultures of *Mm. albus* BG8 grown with copper and either OB3b-MB or SB2-MB



**Fig. 6 Gene expression in *Mc. capsulatus* Bath.** RT-qPCR of **A** *mbnT-Bath*, **B** *mmoX*, **C** *pmoA*, and **D** *mopE* in *Mc. capsulatus* Bath grown with or without 1  $\mu$ M Cu and 5  $\mu$ M MB. Error bar indicates the range of biological duplicate or triplicate samples. Letter over bars indicates no significant differences determined by Tukey's honestly significant difference test ( $p < 0.05$ ).

(Fig. 2A). Expression of various genes involved either in copper storage (*csp3*), carbon oxidation (*pmoA* and *mxoF*), or putative MB uptake (*mbnT-BG8*) was not significantly affected by the addition of either type of MB (Fig. 3). Growth of the *Mm. albus* BG8  $\Delta$ *mbnT* mutant was comparable to that of wild type under all conditions tested (Fig. 1B, S5B). Copper uptake by *Mm. albus* BG8  $\Delta$ *mbnT* was also not affected by the addition of either form of MB, nor was expression of various genes involved in methane oxidation or copper storage (Figs. 2B, 4).

The addition of 5  $\mu$ M TRIEN in the presence of 1  $\mu$ M copper significantly inhibited the growth of *Mm. albus* BG8 wild type as compared to growth in the presence of copper alone (Figs. S5A and S6A). The addition of either form of MB did not improve the growth of *Mm. albus* BG8 wild type in the presence of copper and TRIEN (Figs. S5A and S6A). Expression of various genes involved in methane/methanol oxidation (*pmoA*, *mxoF*) or copper storage (*csp3*) was not significantly affected in *Mm. albus* BG8 grown in the presence of TRIEN, copper, and/or either form of MB (Fig. S7), nor was copper uptake (Fig. S8A). The addition of OB3b-MB in conjunction with TRIEN did reduce the growth of *Mm. albus* BG8  $\Delta$ *mbnT* as compared to the presence of copper alone or copper plus TRIEN (Figs. S5B and S6B). Expression of various genes involved in methane/methanol oxidation (*pmoA*, *mxoF*) or copper storage (*csp3*) was not significantly affected in *Mm. albus* BG8  $\Delta$ *mbnT* (Fig. S9), nor was copper uptake when the mutant was grown in the presence of TRIEN with or without either form of MB, although the mutant collected more copper in the presence of SB2-MB vs. OB3b-MB (Fig. S8B).

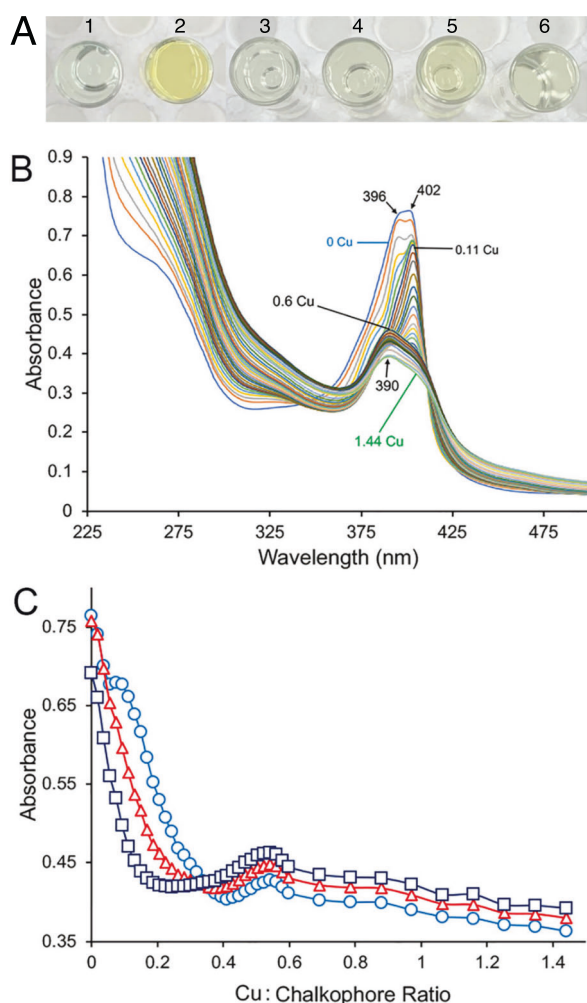
#### Growth of *Methylocystis* sp. strain Rockwell in the presence of varying amounts of copper, MB and TRIEN

Similar to *Mm. albus* BG8, *Methylocystis* sp. strain Rockwell cannot express sMMO, and its growth was inhibited in the absence of

copper as compared to the presence of 1  $\mu$ M copper (Fig. 1C, S5C). Addition of SB2-MB in the presence of copper did not affect the growth of *Methylocystis* sp. strain Rockwell, whereas OB3b-MB significantly reduced growth (Fig. 1C, S5C). Expression of various genes involved in carbon assimilation (*pmoA*, *mxoF*), copper storage (*csp1*), or putative MB uptake (*mbnT1-Rockwell*, *mbnT2-Rockwell*) in *Methylocystis* sp. strain Rockwell was not affected by the addition of either form of MB (Fig. 5). Overall, 5  $\mu$ M TRIEN significantly inhibited the growth of *Methylocystis* sp. strain Rockwell in the presence of 1  $\mu$ M copper, which was resolved only in the presence of 5  $\mu$ M SB2-MB (Figs. S5C and S6C). Copper uptake by *Methylocystis* sp. strain Rockwell was significantly reduced in the presence of OB3b-MB, but not in the presence of SB2-MB, regardless if TRIEN was present or not (Fig. 2C, S8C). Expression of *mbnT1-Rockwell* and *csp1* of *Methylocystis* sp. strain Rockwell increased in the presence of copper, TRIEN, and OB3b-MB as compared to that under 1  $\mu$ M copper while no significant difference was observed for *pmoA*, *mbnT2-Rockwell*, or *mxoF* when comparing these two conditions (Fig. S10).

#### Growth of *Mc. capsulatus* Bath in the presence of varying amounts of copper and methanobactin

*Mc. capsulatus* Bath grows in both the presence and absence of copper as it can express both forms of MMO (Fig. 1D, S5D). Growth was faster and more extensive in the presence of copper, indicating that, as found earlier [51], *Mc. capsulatus* Bath has greater carbon conversion efficiency under pMMO-expressing conditions. Addition of either OB3b-MB or SB2-MB in the presence of copper did not affect growth (Fig. 1D, S5D). Copper uptake by *Mc. capsulatus* Bath was also not affected by the presence of either form of MB (Fig. 2D). Expression of various genes by *Mc. capsulatus* Bath was not affected by the addition of MB including a putative



**Fig. 7** Isolation and characterization of a novel copper-binding compound produced by *Mm. album* BG8. **A** Filtered spent medium of *Mm. album* BG8 grown in the presence of (1) 1  $\mu\text{M}$  copper and (2) 1  $\mu\text{M}$  copper and 5  $\mu\text{M}$  TRIEN, with abiotic controls (3) NMS, (4) NMS, 1  $\mu\text{M}$  copper, and 5  $\mu\text{M}$  TRIEN, (5) NMS and 5  $\mu\text{M}$  OB3b-MB, (6) NMS and 5  $\mu\text{M}$  SB2-MB. **B** UV-Visible absorption spectra of 535 nmol of the chalkophore isolated from *Mm. album* BG8 and following the addition of copper (as  $\text{CuCl}_2$ ) initially in 10 nmol increments (up to 320 nmol) and then in 50 nmol increments (for an additional 450 nmol copper, or 770 nmol copper in total). **C** Absorbance changes at 402 nm (O), 396 nm ( $\Delta$ ), and 390 nm ( $\square$ ) following copper addition. Numbers in **B** refer to mole ratio of copper to *Mm. album* BG8 chalkophore.

MB uptake system (*mbnT-Bath*; Fig. 6). Only the presence/absence of copper had any significant effect on gene expression, and then only on *mmoX* (encoding for a subunit of the sMMO) and *mopE* (encoding for a copper uptake protein). Activity of sMMO was also not affected by the presence of either form of MB, i.e., activity via the naphthalene assay was only evident in the absence of copper (Fig. S11).

#### Localization of MB via Immunoblotting in *Mm. album* BG8

To determine if methanotrophs can take up foreign MB, immunoblotting assays were first performed. Monoclonal antibodies were successfully raised to OB3b-MB, but repeated attempts to generate high-affinity antibodies in rats and mice were unsuccessful for SB2-MB (data not shown). Control immunoblots showed successful monoclonal antibody hybridization to OB3b-MB, but not to lysozyme or *E. coli* cell extracts (Fig. S12). Monoclonal OB3b-MB antibody (10B10), however, cross-

hybridized with cell extracts of *Mm. album* BG8 grown in the presence of 1  $\mu\text{M}$  copper and absence of OB3b-MB over two growth cycles. Greater hybridization to *Mm. album* BG8 cell extract was observed in the presence of 1  $\mu\text{M}$  copper + 5  $\mu\text{M}$  OB3b-MB than in the absence of OB3b-MB (Fig. S12), but very little hybridization was observed in the spent medium or wash buffer when *Mm. album* BG8 was grown in the presence of OB3b-MB (Fig. S12). These data suggest that *Mm. album* BG8 produces some compound analogous to OB3b-MB, but this methanotroph also takes up OB3b-MB as evidenced by greater hybridization signal in the cell extract and low signal in the spent medium and wash buffer when *Mm. album* BG8 was grown in the presence of OB3b-MB. Due to the evidence of cross-hybridization of monoclonal OB3b-MB antibodies in *Mm. album* BG8 and the inability to raise monoclonal antibodies to SB2-MB, these experiments were not replicated in other methanotrophs.

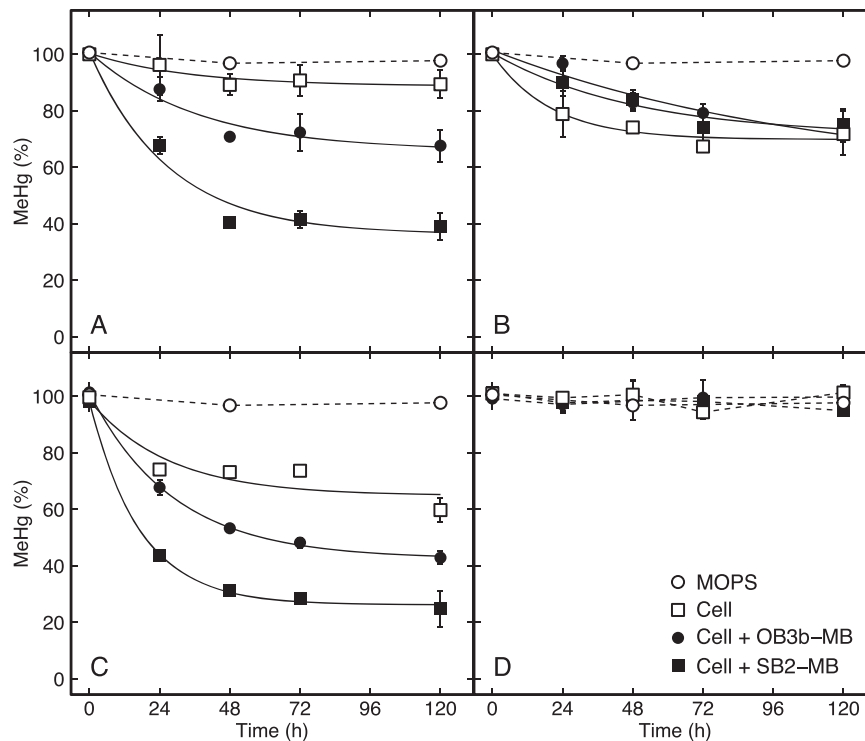
#### Evidence of a novel chalkophore from *Mm. album* BG8

Given that neither form of MB had any measurable effect on *Mm. album* BG8 wild type or the  $\Delta\text{mbnT}$  mutant and the monoclonal OB3b-MB antibody cross-hybridized to cell extracts of *Mm. album* BG8, the possibility that *Mm. album* BG8 makes some copper-binding compound was investigated further. Earlier efforts indicated that *Mm. album* BG8 does secrete some sort of chalkophore, but under standard growth conditions produces very little of it, making characterization difficult [52]. *Mm. album* BG8 was grown in the presence of TRIEN, a strong abiotic chelator of copper, to determine if copper limitation could induce the production of this chalkophore. When *Mm. album* BG8 was grown in the presence of 1  $\mu\text{M}$  copper + 5  $\mu\text{M}$  TRIEN, growth was visibly reduced (Figs. S5A and S6A) and the spent medium became yellow (Fig. 7A). Such coloration was not observed when *Mm. album* BG8 was grown in the presence of copper, indicating that *Mm. album* BG8 secretes some yellowish substance when copper availability is reduced through the addition of TRIEN. This putative chalkophore was found to have a molecular mass of 649.95 (Fig. S13A) or 653.29 Da (Fig. S13B) as determined by MALDI-TOF or ESI-MS, respectively. Following the addition of  $\text{CuCl}_2$  the molecular mass shifted to 711.35 (Fig. S13A) and 713.35 Da (Fig. S13C) as determined by MALDI-TOF or ESI-MS, indicating that this substance indeed binds copper (i.e., is a chalkophore), but likely loses 2 or  $3\text{H}^+$  after doing so (Fig. S13). The UV-VIS spectrum of the isolated chalkophore did not have the characteristic peaks present in MBs (i.e., at  $\sim 340$  and  $394$  nm), but did exhibit distinct absorption maxima at 396 and 402 nm with a molar extinction coefficient of  $1.6\text{ mM}^{-1}\text{ cm}^{-1}$  at 402 nm (Fig. 7B, C, S14A). A discrete isosbestic point at 340 nm at mole ratios of copper:chalkophore between 0 and 0.19 was evident (Fig. S14A). Absorption maxima shifted to 404 nm at copper:chalkophore ratios between 0.2 and 0.45 (Fig. 7, S14B) with a discrete isosbestic point at 398 and to 390 nm at Cu to chalkophore ratios above 0.6 (Fig. 7, S14C).

No such coloration in the spent medium was observed for *Methylocystis* sp. strain Rockwell under any condition (data not shown). Attempts to identify novel chalkophore(s) in *Mc. capsulatus* Bath were not pursued as this strain has already been shown to produce membrane-bound and secreted copper-binding polypeptides (i.e., MopE and MopE\*, respectively) [23–26].

#### Effect of MB on methylmercury degradation by *Mm. album* BG8 wild type, $\Delta\text{mbnT}$ mutant, *Methylocystis* sp. strain Rockwell, and *Mc. capsulatus* Bath

Given the uncertainty of the immunoblot data and evidence that *Mm. album* BG8 produces a competitive chalkophore, to determine if MB theft occurs between methanotrophs, demethylation of MeHg by various strains was monitored in the absence or presence of OB3b-MB and SB2-MB (Fig. 8). This was done as earlier work has shown methanotrophs expressing and taking up MB can



**Fig. 8 Degradation of methylmercury (MeHg) by different methanotrophs.** **A** *Mm. album* BG8 wild type, **B** *Mm. album* BG8  $\Delta mbnT$ , **C** *Methylocystis* sp. strain Rockwell, and **D** *Mc. capsulatus* Bath in MOPS buffer (5 mM). Degradation was fitted to an exponential decay model with stabilization over time (solid line). The total added MeHg, methanobactin (MB), and cell concentrations were 5 nM, 45  $\mu\text{M}$ , and  $10^8$  cells  $\text{mL}^{-1}$  at  $t = 0$  h. Error bars represent the standard deviation of at least biological duplicates. Where error bars are not visible, symbol size is greater than the measured standard deviation.

degrade significant amounts of MeHg [34]. That is, MB appears to serve as a device to deliver MeHg inside the cell where it is degraded, but not by the well-known organomercurial lyase as these microbes lack *merB*. Rather, data suggest that MeHg degradation may be carried out by the periplasmic methanol dehydrogenase that all methanotrophs possess [34]. If *Mm. album* BG8, *Methylocystis* sp. strain Rockwell, and/or *Mc. capsulatus* Bath can take up MB, one would expect that these methanotrophs would be able to degrade MeHg in the presence of MB but not in its absence. Relatively little MeHg degradation was observed in *Mm. album* BG8 in the absence of MB (~10%), but this increased in the presence of both OB3b-MB and SB2-MB (32 and 61%, respectively; Fig. 8). In the absence of either MB, MeHg degradation was observed in *Methylocystis* sp. strain Rockwell (40%), and degradation increased in the presence of both OB3b-MB and SB2-MB (57 and 75%, respectively). Interestingly, under no condition was MeHg degradation observed in *Mc. capsulatus* Bath, nor was the degradation of MeHg by the  $\Delta mbnT$  mutant of *Mm. album* BG8 significantly different in the presence or absence of either form of MB (Fig. 8).

## DISCUSSION

It is well-known that microorganisms have active “social lives”, i.e., microbes exhibit a range of behaviors ranging from cooperation, competition, and cheating [53–55]. An example is the sharing of siderophores amongst microorganisms to meet iron requirements. Iron availability commonly limits microbial growth due to the insolubility of Fe(III), and many microbes produce siderophores for iron solubilization and collection [56, 57]. Given that these compounds are secreted, they can be considered “public goods”, i.e., they are costly for an individual microorganism to make, but can be utilized by other microbes for iron collection [53–55]. A challenge that then arises is that microbes can and do develop

cheating strategies, i.e., some microorganisms with the inability to produce siderophores steal them to meet their needs and such cheating strategies likely play important roles in the diversification and evolution of microbial communities [30–33].

Previous studies suggested that methanotrophs do not utilize a public good for copper collection, i.e., it was shown earlier that *Ms. trichosporium* OB3b outcompetes *Mm. album* BG8 for copper, and thus predominates in mixed cultures [58]. This conclusion, however, appears to be overstated as the *Mm. album* BG8 was unequivocally present in large numbers in these experiments, suggesting that they have some mechanism(s) to collect copper in the presence of MB-expressing methanotrophs. Although *Mm. album* BG8 does not have genes for MB production, it does express something akin to MB, i.e., it secretes a copper-binding compound, especially under copper-limiting conditions, that appears to compete with MBs for copper. Further, the *Mm. album* BG8  $\Delta mbnT$  mutant exhibited a wild-type phenotype, indicating that the chalkophore expressed by *Mm. album* BG8 is effective in competing for copper in the presence of MB. Nonetheless, multiple data sets indicate that *Mm. album* BG8, in addition to being able to effectively compete with MB by producing a novel chalkophore, also engages in MB theft. First, although TRIEN affected growth of both *Mm. album* BG8 wild type and  $\Delta mbnT$  mutant, growth of *Mm. album* BG8 wild type was not further affected by the concurrent addition of OB3b-MB, but that of the  $\Delta mbnT$  mutant was. Second, MB enhanced MeHg demethylation in *Mm. album* BG8 wild type, but not the  $\Delta mbnT$  mutant.

We were unable to identify any novel chalkophore produced by *Methylocystis* sp. strain Rockwell, but it does appear to act as a cheater by taking up MB—preferentially Group II MB—to meet its copper requirements as growth and copper uptake was inhibited in the presence of OB3b-MB (a group I MB), but not SB2-MB (a Group II MB). This conclusion is supported by finding that the addition of TRIEN inhibited the growth of *Methylocystis* sp. strain



Rockwell, but concurrent addition of SB2-MB relieved such inhibition. Although we could not directly determine SB2-MB uptake via immunoblots as we were unsuccessful in raising monoclonal antibodies to SB2-MB, MeHg degradation data also indicate MB can be taken up by *Methylocystis* sp. strain Rockwell. On the other hand, *Mc. capsulatus* Bath does not appear to steal MB as its genome had no genes with high similarity to *mbnT* and the addition of MB did not enable this methanotroph to degrade MeHg. Rather, *Mc. capsulatus* Bath appears to have an effective strategy to compete for copper in the presence of MB (i.e., MopE/MopE\*), as the addition of MB had no effect on its growth, copper uptake, and gene expression. Thus, MB may serve as a sort of public good to some methanotrophs, but is not of benefit to all methanotrophs. Given that methanotrophs use a variety of strategies to collect copper, these interactions likely are significant in structuring methanotrophic communities in situ.

While herein, we report methanotrophic interactions based on competition for copper, including MB theft, interspecies interactions have been documented earlier for methanotrophs, e.g., recognition of and response to acyl-homoserine lactone receptor/transcription factors and uptake of foreign MB by species that can make MB. Such interactions, however were within species of the same family [59, 60]. Here we show interactions not only between members of the same class of methanotrophs (i.e., Alphaproteobacteria), but also between members of different classes (i.e., Alpha- vs. Gammaproteobacteria methanotrophs), indicating methanotrophic interactions can be phylogenetically far-ranging. It may be that uptake of MB from the environment by non-MB-producing methanotrophs not only enhances their ability to collect copper, but also gives them an advantage by acting as a MB sink, thereby placing MB-producing methanotrophs at a disadvantage. Such a finding lends support to the hypothesis that as competition for resources becomes more local, the influence of species relatedness for cooperation is reduced, thus decreasing altruism and allowing cheating to become more pronounced [53–55]. That is, as methanotrophs of different phylogenies co-habitat, kin recognition/discrimination becomes less effective for these microbes and MB can be more readily stolen. These findings raise the intriguing question as what prevents MB stealers from overwhelming the population? As suggested for siderophores, it may be that MB production and distribution to non-MB-producing methanotrophs provides both direct and indirect fitness benefits to MB-producers that outweigh the costs of MB synthesis and loss. The magnitude and distribution of such benefits, however, are likely to be highly dependent on environmental conditions (e.g., copper availability) and population density as suggested for siderophores [53].

Finally, the finding that the heterologous uptake or theft of MB enables MB non-producers to detoxify a highly toxic organic form of mercury suggests that methanotrophic-mediated MeHg detoxification may be more widespread than previously thought in the natural environment. Examining this in more detail will likely be very informative and will serve as key inputs for metabolic and reactive transport models that can be used to better predict net MeHg production and Hg biogeochemical cycling in the environment.

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## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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