A TonB-dependent Transporter is Responsible for Methanobactin Uptake by *Methylosinus trichosporium* OB3b

By

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Running title: Methanobactin uptake by methanotrophs

ABSTRACT

Methanobactin, a small modified polypeptide synthesized by methanotrophs for copper uptake, has been found to be chromosomally encoded. The gene encoding for the polypeptide precursor of methanobactin, *mbnA*, is part of a gene cluster that also includes several genes encoding for proteins of unknown function (but speculated to be involved in methanobactin formation), as well as *mbnT*, encoding for a TonB-dependent transporter hypothesized to be responsible for methanobactin uptake. To determine if *mbnT* was truly responsible for methanobactin uptake, a knock-out 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 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 for polypeptides of the soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO), respectively, also changed significantly when methanobactin was added, indicating that the mutant was unable to collect copper under these conditions. Copper uptake and gene expression, however, was not affected in *M. trichosporium* OB3b wildtype, 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 *M. trichosporium* OB3b wildtype, indicating that this methanotroph has multiple mechanisms for copper uptake.

45 **INTRODUCTION**

46 Methanotrophs, or methane-oxidizing bacteria, are a group of microbes with great
47 environmental and industrial importance. For example, methanotrophs are well known to play
48 a key role in controlling the net emission of methane from soils, a potent greenhouse gas with a
49 global warming potential ~34x that of carbon dioxide over a 100-year time frame (1). In fact, it
50 is estimated that as much as 90% of methane generated in anaerobic soils via methanogenesis
51 may be removed via methanotrophy (2). Further, methanotrophs oxidize methane under
52 ambient temperatures and pressures, and thus are attractive platforms for the valorization of
53 methane to products such as single-cell protein, bioplastics, biofuels, and osmo-protectants (3-
54 5).

55

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

63

64 A key issue affecting aerobic methanotrophic activity, particularly those in the
65 *Gammaproteobacteria* and *Alphaproteobacteria*, is the availability of copper. It was first
66 discovered over 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 also has relatively poor affinity for methane. (4, 13-16). pMMO, conversely, has a relatively narrow substrate range and 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 (< 1200 Da) with two heterocyclic rings, either an imidazole, oxazolone or pyrazinedione ring, each with an associated enethiol group that together are responsible for copper binding (19-22). Biochemical analyses indicated that methanobactin could be formed from a polypeptide precursor with the heterocyclic rings derived from a -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 indeed is the precursor of methanobactin and that it is part of a gene cluster (Figure 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 for 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

Methylosinus trichosporium OB3b wildtype 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 side-arm Erlenmeyer flasks with 30-50 ml of medium shaken at 200 rpm. Copper (as $CuCl_2$) and methanobactin from *M. trichosporium* OB3b were filter-sterilized and 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 3-12-hour intervals. Cultures were grown in at least duplicate biological replicates and 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,* encoding for a TonB-dependent transporter using the protocol described in Semrau, et al., 2013 (23). Briefly 3' and 5' DNA regions of *mbnT* (Arm A and B, respectively) were selectively amplified by PCR using primers listed in Table 1. These PCR products were then digested with *Bam*HI, separated by gel electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Arms A and B were ligated and again PCR-amplified. The amplified product was digested with *Eco*RI and *Hind*III and inserted into pK18mobsacB yielding construct pWG01. The gentamycin resistance gene (Gm^R) was then excised from plasmid p34S-Gm using *Bam*HI. This was then inserted into the *Bam*HI site between Arm 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 onto NMS plates with 2.5 µg• ml^{-1} gentamycin. 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 µg• ml^{-1} gentamycin and 10 µg• ml^{-1} nalidixic acid. Successful knockout of *mbnT* via double homologous recombination was confirmed by screening of kanamycin-sensitive and sucrose-resistant phenotype, PCR, and sequencing.

RNA extraction and reverse transcription

RNA was isolated using the method described earlier (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 re-suspended in 0.75 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 1% (w/v) hexadecyltrimethylammonium bromide (CTAB)] before lysis using 20 % SDS, 20% lauryl sarkosine, 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 ND1000; NanoDrop Technologies, Inc., Wilmington, DE). RNA samples were stored at -80 °C and 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 of mRNA to cDNA (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR analyses were performed to determine the relative expression of the *pmoA*, *mmoX*, and *mbnA* genes in *M. trichosporium* OB3b and *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 whose specificity had been verified by sequencing and gel electrophoresis. Measurements were performed in 96-well reaction PCR plates using CFX Connect Real Time PCR Detection System (Bio-Rad, Hercules CA). In each well, qPCR reaction (20 µl) consisted of 0.8 µl cDNA, 1 x iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules CA), 0.5 µM of each of 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

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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 temperature ranging from 55 °C to 95 °C after the completion of amplification cycles. The threshold amplification cycle (Ct) values were then imported from CFX Manager Software (Bio-Rad) into Microsoft Excel to quantify the relative expression of different genes. The comparative Ct 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 *M. trichosporium* OB3b wild-type and *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 re-suspended in 1mL 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 incubated for 2 hours at 95°C with inversion every 20 min. Copper associated with biomass was subsequently analyzed using an inductively coupled plasma mass spectrometry (Agilent Technologies, Santa Clara, CA). At least duplicate biological samples for every condition were analyzed.

Methanobactin in spent media and in cell-extracts

For characterization of the location of methanobactin from *M. trichosporium* OB3b wildtype and *mbnT*::Gm^R mutant, cells were cultured in 12 l of NMS medium amended with 0.2 µM $CuCl_2$ in a 15 l New Brunswick fermenter at 30°C for 48 h. Following the incubation period, 10 l of the

177 culture was removed and 10 l of fresh NMS media was added to the fermenter and the copper
178 concentration increased to 5 µM. This sequence was then repeated with increasing copper
179 concentration to 10 and 20 µM in subsequent fermenter turnovers.
180
181 The extracellular fraction and cells from each 10 l sample were separated via tangential flow
182 filtration using a 10,000 Da molecular mass filter as previously described (30). The cells from
183 the retentate were then harvested by centrifugation at 13,200 x g at 4°C. The pellet was re-
184 suspended in 10 mM phosphate buffer, pH 7.3 and centrifuged at 13,200 x g at 4°C. This cell
185 pellet was then re-suspended in a minimal volume of 10 mM phosphate buffer, at a pH of 6.8
186 plus 1µg DNAase • ml^{-1} and lysed by three passes through an EmlusiFex C3 high-pressure
187 homogenizer at 15,000 psi (Avestin. Inc. Ottawa, ON, Canada) at 4°C. The cell extract was then
188 centrifuged at 13,000 x g for 20 min to remove un-lysed cells followed by filtration though 0.2
189 µm Millipore filters (Billerica, MA).
190
191 **Methanobactin antibody generation**
192 Antibodies to methanobactin from *M. trichosporium* OB3b were produced in Lou/c rats which
193 were immunized subcutaneously and intraperitoneally with a methanobactin-ovalbumine
194 fusion protein (50 µg), 5 nmol CPG oligonucleotide (Tib Molbiol, Berlin), 500 µl phosphate
195 buffered saline and 500 µl incomplete Freund's adjuvant. A boost without adjuvant was given
196 six weeks after the primary injection. Tissue culture supernatants (TCS) were tested in a solid-
197 phase immunoassay with methanobactin coupled to BSA or an irrelevant peptide coupled to
198 BSA coated ELISA plates at a concentration of 4 µg• ml^{-1}. Antibodies (mAb) from TCS bound to

methanobactin were detected with horseradish peroxidase conjugated mAbs against the rat IgG isotypes (TIB173 IgG2a, TIB174 IgG2b, TIB170 IgG1 all from ATCC, R-2c IgG2c homemade), thus avoiding mAbs of IgM class. HRP was visualized with ready to use 3,3',5,5'-tetramethylbenzidine (TMB; 1-StepTM Ultra TMB-ELISA, ThermoFisher, Waltham, MA). Hybridomas that reacted specifically with methanobactin were frozen and the antibody containing TCS were 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, 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-PAA-PVDF membranes).

Chemiluminescence western dot-blots

DITC-PAA-PVDF transfer membranes were sized to fit an 8 x 12 well Bio-Rad Bio-Dot dot blot (Bio-Rad Inc. Hercules, CA), washed with 20mM Tris-HCl plus 0.5M NaCl (TBS) at pH 7.5, and loaded with filter paper onto this dot blotter. Samples (spent media and cell extracts) were then loaded under vacuum and dried for 30 min under vacuum. The membrane was then

wetted with 50% CH_3OH:50% H_2O and washed twice in TBS at room temperature. The membrane was then incubated overnight in 0.2% nonfat dry milk in TBS overnight at 4°C, with the TBS subsequently decanted. The membrane was then re-suspended in TBS plus 0.1% Tween 20 (TTBS) at pH 7.5 and incubated for 10 minutes 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 re-suspended 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, the membrane re-suspended 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 re-suspended 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 washed three times with TTBS as described above. Visualization of the blot was done via Bio Rad Immun-Star AP substrate for chemiluminescence (Hercules, CA, USA) following manufacturer's suggested procedure.

RESULTS

Using marker-exchange protocols, a transconjugant colony with a double homologous recombination event in which *mbnT* was successfully knocked out was identified (Figure 2). This was confirmed by sequencing, as well as verification of the mutant being gentamycin 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 *M. trichosporium* wildtype. When grown in varying copper concentrations, both *mbnT*::Gm^R mutant and wildtype had increasing amounts of copper associated with biomass (Figure 3A). Further, gene expression in both mutant and wildtype 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 (Figure 3B, C). Finally, expression of *mbnA*, encoding for the precursor polypeptide of methanobactin, decreased substantially in both in *M. trichosporium* OB3b wildtype and *mbnT*::Gm^R mutant as copper increased, indicating that the knock-out of *mbnT* did not affect methanobactin expression (Figure 3D).

These findings suggest that either *mbnT* is not involved in copper uptake (i.e., binding of copper-methanobactin complexes) or 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 wildtype strain of *M. trichosporium* OB3b was assayed for a wide range of copper concentrations using immuno-blotting assays. As shown in Figure 4, as the growth concentration of copper increased, the amount of methanobactin in the spent medium decreased in *M. trichosporium* OB3b wildtype, 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 wildtype strain of *M. trichosporium* OB3b were then grown in the presence of 1 µM copper and varying amounts of copper-free methanobactin. As shown in Figure 5A, in the presence of either 5 or 50 µM methanobactin, copper associated with biomass of the *mbnT*::Gm^R mutant decreased over three-fold, while no significant change in copper levels of *M. trichosporium* OB3b wildtype was observed. Further, expression of *mmoX* increased over three orders of magnitude in the *mbnT*::Gm^R mutant while *pmoA* expression dropped by approximately eight-fold. No significant change in expression of either *mmoX* or *pmoA* was observed, however, in *M. trichosporium* OB3b wildtype (Figure 5B, C). Collectively, these data show that in the presence of molar excess of methanobactin, copper was still bioavailable to *M. trichosporium* OB3b wildtype, but was not for the *mbnT*::Gm^R mutant. Additionally, it was assayed if the addition of exogenous methanobactin affected *mbnA* expression in *M. trichosporium* OB3b wildtype and *mbnT*::Gm^R mutant. As shown in Figure 5D, as increasing amounts of methanobactin were added, *mbnA* expression increased in both wildtype 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: (1) methanobactin could be taken up by *M. trichosporium* OB3b wildtype but not the *mbnT*::Gm^R mutant, and; (2) the *mbnT*::Gm^R mutant of *M. trichosporium* OB3b was unable to take up copper if methanobactin was exogenously added to bind copper, but *M. trichosporium* OB3b wildtype was.

The data also show, however, *M. trichosporium* OB3b has an alternative mechanism(s) for copper uptake, i.e., in the absence of any exogenous methanobactin, the amount of copper in the wildtype and *mbnT*::Gm^R strains of *M. trichosporium* OB3b was 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 when compared to 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 for 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 preceded by *mbnR* and *mbnI,* encoding for 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 ferri-siderophore, transmitting a signal to a membrane sensor that then activates an

extracytoplasmic function sigma factor. This ultimately induces expression of genes required for siderophore synthesis, as well as in some cases genes unrelated to siderophore production or uptake, e.g., genes encoding for exotoxins and proteases (e.g., 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 is 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 expression of *mbnA*. That is, *mbnA* expression in both *M. trichosporium* OB3b wildtype and the *mbnT*::Gm^R mutant decreased significantly with increasing copper, but the magnitude of the drop in expression was greater in wildtype (Figure 3D). Further, in the presence of 1 µM copper and varying amounts of exogenous methanobactin, *mbnA* expression in *M. trichosporium* OB3b wildtype and *mbnT*::Gm^R mutant responded with the same pattern (Figure 5D). It appears that another regulatory circuit is involved in controlling 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 knock-out of *mbnT*, and show that this is responsible for methanobactin uptake. The phenotype of the *mbnT*::Gm^R mutant, however,

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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 both in *M. trichosporium* OB3b wildtype and 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, an outer membrane protein, CorA, exists 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* that both bind Cu(II) (45-47). A gene encoding for 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 varying 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.

FUNDING INFORMATION

353 This research was supported by the Office of Science (Biological and Environmental Research),
354 U.S. Department of Energy, Grant #DE-SC0006630 to JDS and AAD. The funders had no role in
355 study design, data collection and interpretation, or the decision to submit the work for
356 publication.

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359 **REFERENCES**

360 1. IPCC (Intergovernmental Panel on Climate Change) - Fifth Assessment Report, Chapter 8.
361 Anthropogenic and Natural Radiative Forcing. Myhre G, Shindell D, Breon F-M, Collins W,
362 Fuglestvedt J, Huang J, Koch D, Lamarque JF, Lee D, Mendoza B, Nakajima T, Robock A,
363 Stephens G, Takamura T, Zhang H. Edited by TF Stocker, D Qin, GK Plattner, MMB Tignor,
364 SK Allen, J Boschung, A Nauels, Y Xia, V Bex, and PM Midgley. 2013. **8**: 659-740.
365 2. Chowdury TR, Dick RP. 2013. Ecology of aerobic methanotrophs in controlling methane
366 fluxes from wetlands. Appl Soil Ecol. **65**: 8-22.
367 3. Khmelenina VN, ON Rozova, SY But, II Mustakhimov, AS Reshetnikov, AP Beschastnyl,
368 Trotsenko YA. 2015. Biosynthesis of secondary metabolites in methanotrophs:
369 biochemical and genetic aspects (review). Appl Biochem Microbiol. (Russian) **51**: 150-
370 158.
371 4. Semrau JD, DiSpirito AA, Yoon S. 2010. Methanotrophs and copper. FEMS Microbiol Rev
372 **34**: 496-531.
373 5. Strong PJ, S Xie, Clarke WP. 2015. Methane as a resource: can the methanotrophs add
374 value? *Environ. Sci. Technol*. **49**: 4001-4008.
375 6. Op den Camp H, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MSM,
376 Birkeland N-K, Pol A, Dunfield PF. 2009. Environmental, genomic and taxonomic
377 perspectives on methanotrophic *Verrucomicrobia*. Environ Microbiol Rep **1:**293–306.
378 7. Hanson RS, Hanson TE. 1996. Methanotrophic bacteria. Microbiol Rev **60:**439–471.
379 8. Ettwig KF, Butler MK, Le Paslier D, Pelletier E, Mangenot S, Kuypers, MM, Schreiber F,
380 Dutilh BE, Zedelius J, de Beer D, Gloerich J, Wessels, HJ, van Alen T, Luesken F, Wu ML,

van de Pas-Schoonen KT, Op den Camp HJ, Janssen-Megens EM, Francoijs KJ, Stunnenberg H, Weissenbach J, Jetten MS, Strous M. 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464**:543–548.

9. Knittel K, Boetius A. 2009. Anaerobic oxidation of methane: progress with an unknown process. Annu Rev Microbiol **63**:311–334.
10. Haroon MF, Hu S, Imelfort M, Keller J, Hugenholtz P, Yuan Z, Tyson GW. 2013. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature **500**:567–570.
11. Choi D-W, Kunz R, Boyd ES, Semrau JD, Antholine WE, Han J-I, Zahn JA, Boyd JM, de la Mora A, DiSpirito AA. 2003. The membrane-associated methane monooxygenase (pMMO) and pMMO-NADH:quinone oxidoreductase complex from *Methylococcus capsulatus* Bath. J Bacteriol. **185**: 5755-5764.
12. Stanley SH, Prior SD, Leak DJ, Dalton H. 1983. Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane-oxidising organisms: Studies in batch and continuous cultures. Biotechnol Lett **5**: 487-492.
13. Kalyuzhnaya MG, Puri AW, Lidstrom ME. 2015. Metabolic engineering in methanotrophic bacteria. Metabol Eng. **29**: 142-152.
14. Lee SW, DR Keeney, DH Lim, AA DiSpirito, Semrau JD. 2006. Mixed pollutant degradation by *Methylosinus trichosporium* OB3b expressing either soluble or particulate methane monooxygenase: can the tortoise beat the hare? Appl Environ Microbiol. **72**: 7503-7509.
15. Semrau JD. 2011. Bioremediation via methanotrophy: overview of recent findings and suggestions for future research. Front Microbiol. **2**: Article 209.

16. Trotsenko YA, Murrell JC. 2008 Ch. 5. Metabolic aspects of aerobic obligate methanotrophy. *In* Advances in Applied Microbiology, **Vol 63**. pp. 183-229.

17. Lontoh S, Semrau JD. 1998. Methane and trichloroethylene degradation by *Methylosinus trichosporium* expressing particulate methane monooxygenase. Appl Environ Microbiol **64**: 1106-1114.

18. Yoon S, Carey JN, Semrau JD. 2009. Feasibility of atmospheric methane removal using methanotrophic biotrickling filters. Appl Microbiol Biotechnol. **83**:949-956.

19. Behling LA, Hartsel SC, Lewis DE, DiSpirito AA, Choi DW, Masterson LR, Veglia G, Gallagher WH. 2008. NMR, mass spectrometry and chemical evidence reveal a different chemical structure for methanobactin that contains oxazolone rings. J. Amer. Chem. Soc. **130**:12604-12605.

20. El Ghazouani A, Baslé A, Gray J, Graham DW, Firbank SJ, Dennison C. 2012. Variations in methanobactin structure influences copper utilization by methane-oxidizing bacteria. Proc. Natl. Acad. Sci. **109**: 8400-8404.

21. Kim HJ, Graham DW, DiSpirito AA, Alterman MA, Galeva N, Larive CK, Asunskis D, Sherwood PMA. 2004. Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria. Science. **305**: 1612–1615.

22. Krentz BD, Mulheron HJ, Semrau JD, DiSpirito AA, Bandow N, Haft DH, Vuilleumier S, Murrell JC, McEllistrem MT, Hartsel SC, Gallagher W. 2010. A comparison of methanobactins from *Methylosinus trichosporium* OB3b and *Methylocystis* species SB2 predicts they are synthesized from diverse ribosomally produced peptide precursors

424 modified to create a common core for binding and reducing copper ions. Biochemistry.
425 **49**: 10117-10130.
426 23. Semrau JD, Jagadevan S, DiSpirito AA, Khalifa A, Scanlan J, Bergman BH, Freemeier BC,
427 Baral BS, Bandow NS, Vorobev A, Haft DH, Vuilleumier S, Murrell JC. 2013.
428 Methanobactin and MmoD work in concert to act as the "copper-switch" in
429 methanotrophs. Environ Microbiol **15**: 3077-3086.
430 24. Whittenbury R, Phillips KC, Wilkinson JF. 1970. Enrichment, isolation and some
431 properties of methane-utilizing bacteria. J Gen Microbiol **61**: 205-218.
432 25. Vorobev A, Jagadevan S, Baral BS, DiSpirito AA, Freemeier BC, Bergman BH, Bandow NL,
433 Semrau JD. 2013. Detoxification of mercury by methanobactin from *Methylosinus*
434 *trichosporium* OB3b. Appl Environ Microbiol **79**: 5918-5926.
435 26. Simon R. 1984. High frequency mobilization of gram-negative bacterial replicons by the
436 in vitro constructed Tn5-Mob transposon. Molecul Gen Genetics MGG **196**: 413-420.
437 27. Martin H, Murrell JC. 1995. Methane monooxygenase mutants of *Methylosinus*
438 *trichosporium* OB3b constructed by marker-exchange mutagenesis. FEMS Microbiol
439 Lett. **127**: 243–248.
440 28. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C_T
441 method. Nat Protoc **3**: 1101–1108.
442 29. Kalidass B, Ul-Haque MF, Baral BS, DiSpirito AA, Semrau JD. 2015. Competition between
443 metals for binding to methanobactin enables expression of soluble methane
444 monooxygenase in the presence of copper. Appl Environ Microbiol **81**: 1024-2031.

30. Bandow NL, Gallagher WH, Behling L, Choi DW, Semrau JD, Hartsel SC, Gilles VS, Dispirito AA. 2011. Isolation of methanobactin from the spent media of methane-oxidizing bacteria. Meth Enzymol **495**:259-269.

31. Zahn JA, DiSpirito AA. 1996. Membrane-associated methane monooxygenase from *Methylococcus capsulatus* (Bath). J Bacteriol **178**:1018-1029.

32. Rodrigues JA, Combrink J, Brandt WF. 1994. Derivatization of polyvinylidene diflluoride membranes for solid-phase sequencing analysis of a phosphorylated sea urchin embryo histone H1 peptide. Anal Biochem **216**:365 – 372.

33. Balasubramanian R, Smith SM, Rawat S, Yatsunyk LA, Stemmler TL, Rosenzweig AC. 2010. Oxidation of methane by a biological dicopper centre. Nature. **465**: 115-119.

34. Hakemian AS, Kondapalli KC, Tesler J, Hoffman BM, Stemmler TL, Rosenzweig AC. 2008. The metal centers of particulate methane monooxygenase from *Methylosinus trichosporium* OB3b. Biochemistry **47**: 6793-6801.

35. Martinho, M, Choi DW, DiSpirito AA, Antholine WE, Semrau JD, Münck E. 2007. Mössbauer studies of the membrane-associated methane monooxygenase from *Methylococcus capsulatus* Bath: Evidence for a dinuclear iron center. J Am Chem Soc. **129**:15783 – 15785.

36. Kenney GE, Rosenzweig AC. 2013. Genome mining for methanobactins. BMC Biology **11**:17.

37. Braun V, Mahren S, Sauter A. 2006. Gene regulation by transmembrane signaling. BioMetals. **19**: 103-113.

38. Brooks BE, Buchanan SK. 2008. Signaling mechanisms for activation of extracytoplasmic function (ECF) sigma factors. Biochim Biophys Acta. **1778**: 1930-1945.

39. Crosa JH. 1997. Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. Microbiol Mol Biol Rev. **61**: 319-336.

40. Groβe C, Friedrich S, Nies DH. 2007. Contribution of extracytoplasmic function sigma factors to transition metal homeostasis in *Cupriavidus metallidurans* strain CH34. J Mol Microbiol Biotechnol. **12**: 227-240.

41. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. 2002. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aueroginosa*. Proc Natl Acad Sci. **99**: 7072-7077.

42. Mahren S, Braun V. 2003. The FecI extracytoplasmic-function sigman factor of *Escherichia coli* interacts with the β' subunit of RNA polymerase. J Bacteriol. **185**: 1796-1802.

43. Visca P, Leoni L, Wilson MJ, Lamont IL. 2002. Iron transport and regulation, cell signaling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. Mol Microbiol. **45**: 1177-1190.

44. Berson O, Lidstrom ME. 1997. Cloning and characterization of *corA*, a gene encoding for a copper-repressible polypeptide in the type I methanotroph, *Methylomicrobium albus* BG8. FEMS Microbiol Lett. **148**: 169-174.

45. Karlsen OA, Berven FS, Stafford GP, Larsen Ø, Murrell JC, Jensen HB, Fjellbirkeland A. 2003. The surface-associated and secreted MopE protein of *Methylococcus capsulatus*

(Bath) corresponds to changes in the concentration of copper in the growth medium. Appl. Environ. Microbiol. **69**: 2386-2388.

46. Helland R, Fjellbirkeland A, Karlsen OA, Ve T, Lillehaug JR, Jensen HB. 2008. An oxidized tryptophan facilitates copper binding in *Methylococcus capsulatus*-secreted protein MopE. J Biol Chem. **283**: 13897-13904.
47. Ve T, Mathisen K, Helland R, Karlsen OA, Fjellbirkeland A, Røhr Å, Andersson KK, Pedersen R-B, Lillehaug JR, Jensen HB. 2012. The *Methylococcus capsulatus* (Bath) secreted protein, MopE* binds both reduced and oxidized copper. *PLoS One*. e43146.
48. Knapp CW, Fowle DA, Kulczycki E, Roberts JA, Graham DW. 2007. Methane monooxygenase gene expression mediated by methanobactin in the presence of mineral copper sources. Proc Natl Acad Sci USA **104**:12040–12045.

499 Figure 1. Methanobactin gene cluster in *Methylosinus trichosporium* OB3b.

500

501 Figure 2. Verification of knock-out of *mbnT* in *M. trichosporium* by PCR. M: Molecular weight
502 markers; Lane 1: PCR of *mbnT* from *M. trichosporium* OB3b *mbnT*::Gm^R mutant; Lane 2: PCR of
503 *mbnT* from *M. trichosporium* OB3b wildtype; Lane 3, PCR of pK18*mobsacB* backbone in *M.*
504 *trichosporium* OB3b *mbnT*::Gm^R; Lane 4: PCR of pK18*mobsacB* backbone in pWG011.

505

506 Figure 3. Characterization of *M. trichosporium* OB3b wildtype (■) and *mbnT*::Gm^R mutant (□)
507 grown in the presence of varying amounts of copper: (A) Copper associated with biomass; (B)
508 RT-qPCR of *pmoA*; (C) RT-qPCR of *mmoX*; (D) RT-qPCR of *mbnA*. Error bars indicate standard
509 deviation of at least duplicate biological replicates. Indicated *P* values are from one-way
510 analysis of variation (ANOVA).

511

512 Figure 4. Immuno-blotting assays for location of methanobactin in *M. trichosporium* OB3b
513 wildtype and *mbnT*::Gm^R mutant as a function of concentration of copper in the growth medium
514 (0.2, 5, 10 or 20 µM copper). 50 nmol lysozyme (lys) and 50 nmol methanobactin (mb) were
515 used as negative and positive controls, respectively.

516
517 Figure 5. Characterization of *M. trichosporium* OB3b wildtype (■) and *mbnT*::Gm^R mutant (□)
518 grown in the presence of 1 µM copper and varying amounts of methanobactin (MB): (A) Copper
519 associated with biomass; (B). RT-qPCR of *pmoA*; (C) RT-qPCR of *mmoX*; (D) RT-qPCR of *mbnA.*

Error bars indicate standard deviation of at least duplicate biological replicates. Indicated *P* values are from one-way analysis of variation (ANOVA).

Table 1. Primers used in this study.

Primer	Targeted gene	Sequence* (5' – 3')	Reference
Arm A Forward	*mbnT*	ATTTTTgaattcCCAGAAATATGAGATTCCGC[§]	This study
Arm A Reverse		ATTTTTggatccCACGACCAGATCGATGATAC[§]	
Arm B Forward	*mbnT*	ATTTTTggatccTTCGGTTCGATCAACGAGG[§]	This study
Arm B Reverse		ATTTTTaagcttGCCAATCAGCGTGGAGAACC[§]	
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	

*Y, S and R are the IUPAC DNA codes for C/T, C/G and A/G nucleobases respectively.

[§]Lower case letters indicate *Eco*RI, *Bam*HI, or *Hind*III restriction site sequences included in these primers.

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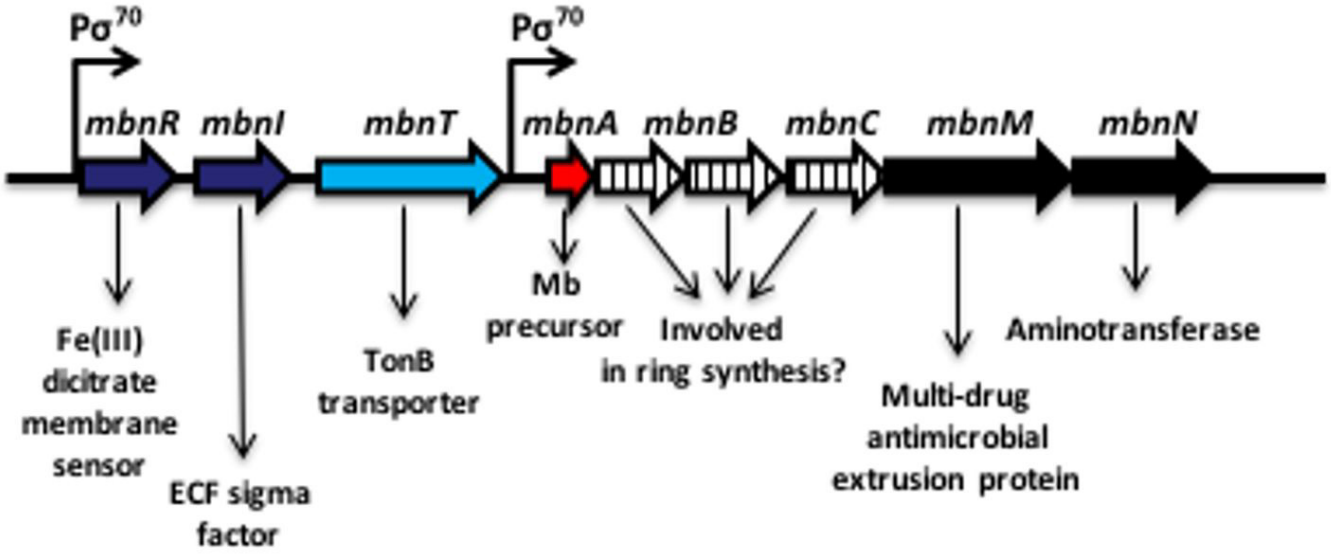

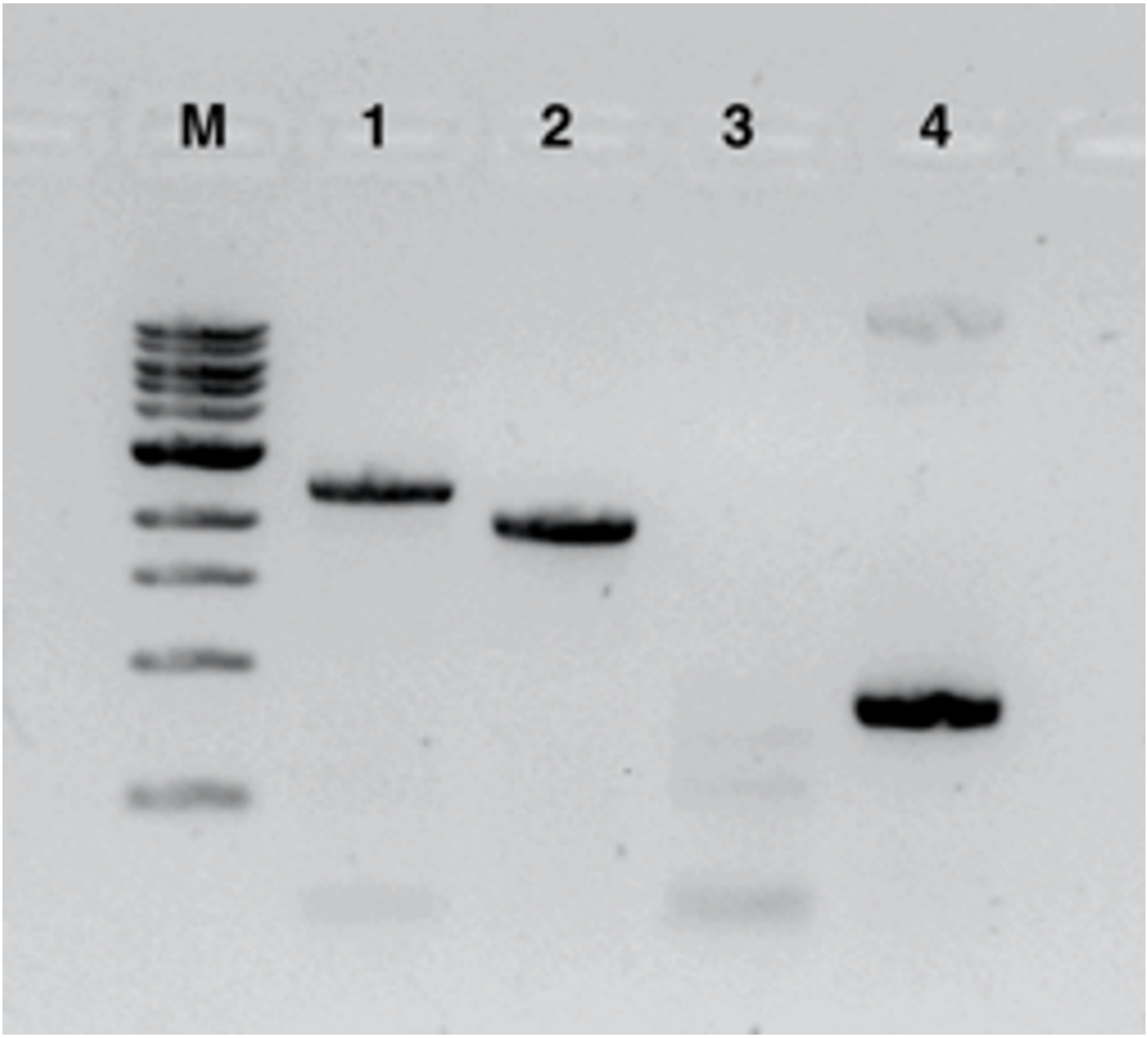

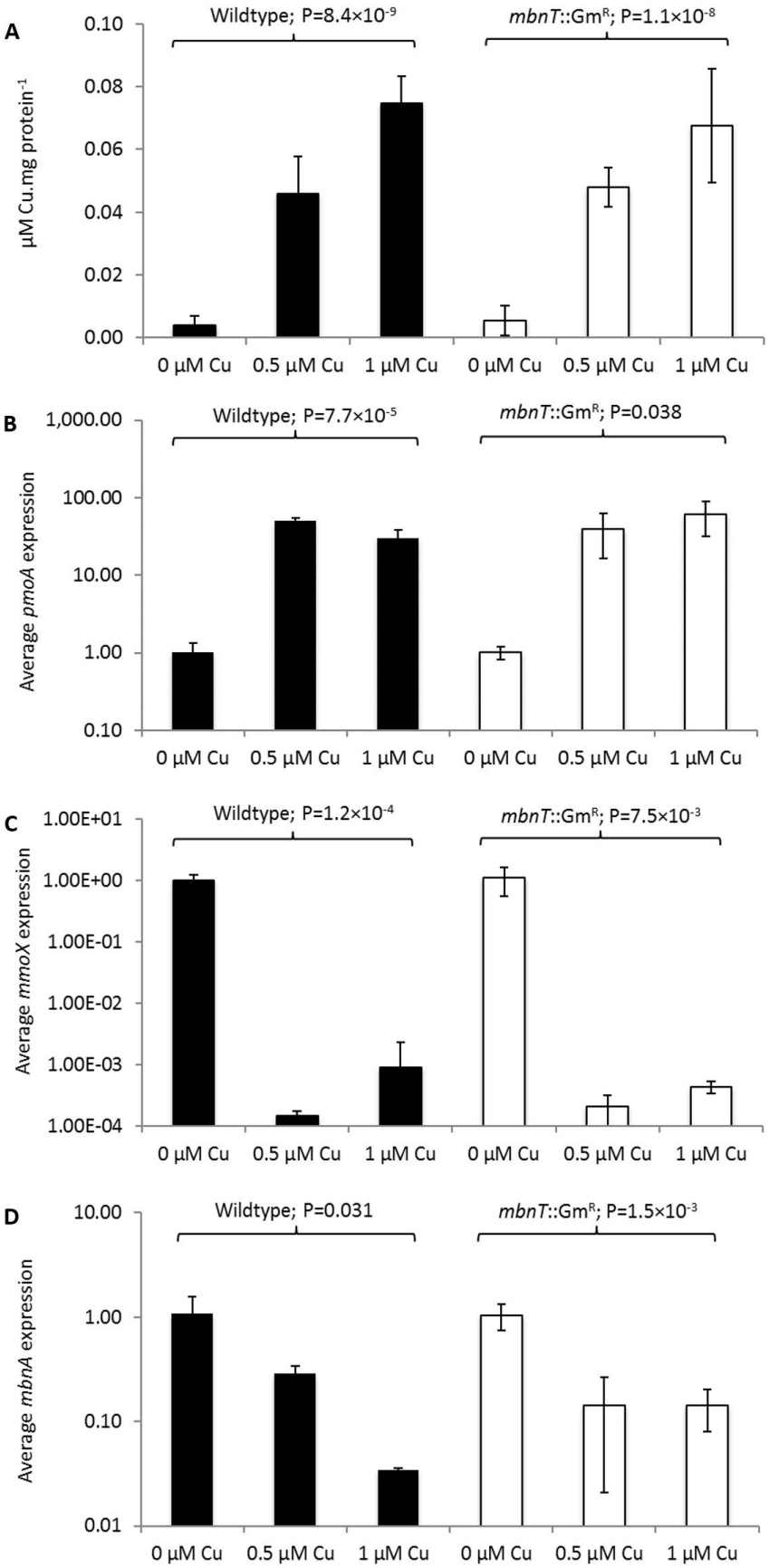

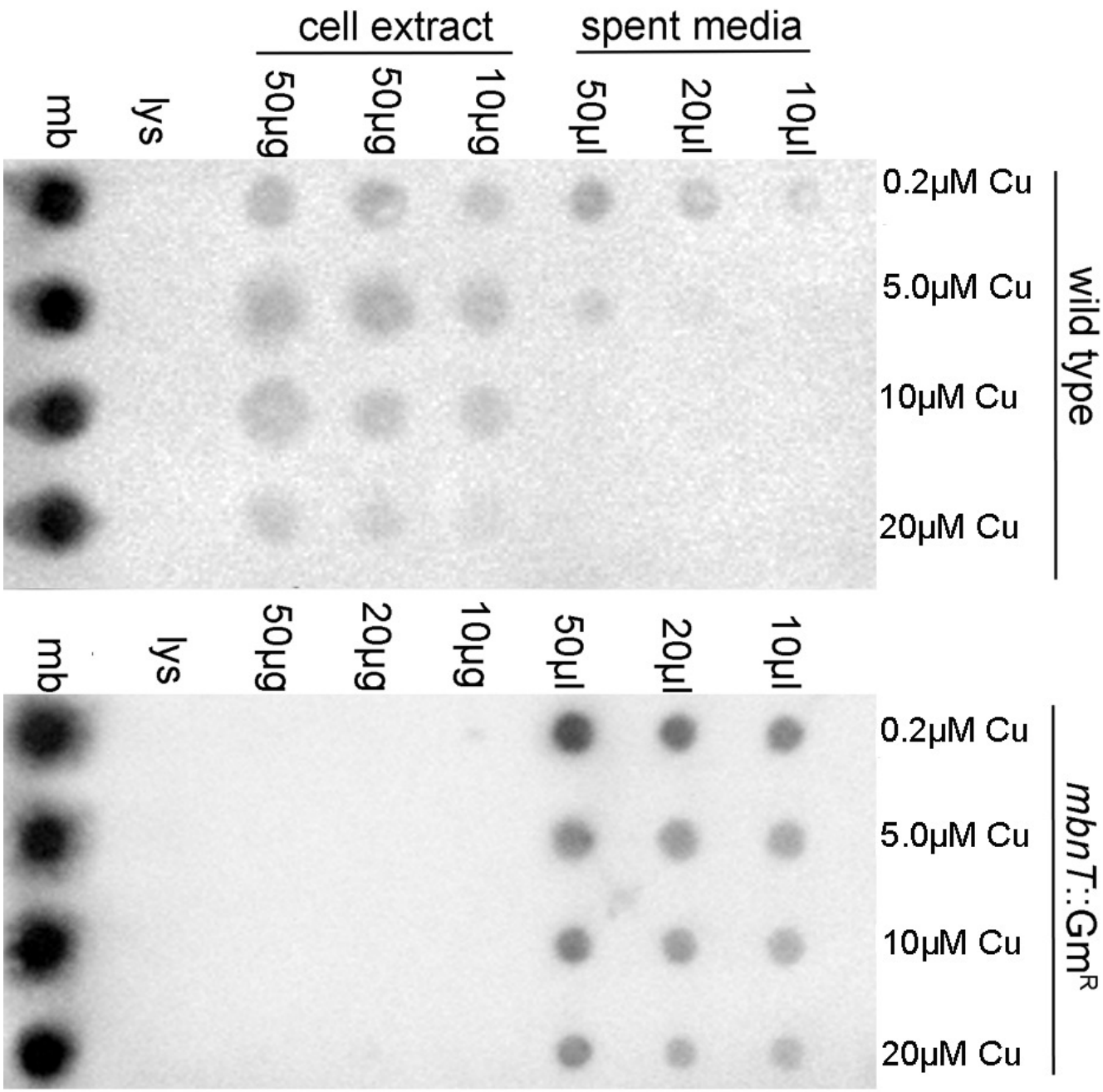
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mbnT::GmR