Methane monooxygenase gene expression mediated by methanobactin in the presence of mineral copper sources

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Methane is a major greenhouse gas linked to global warming; however, patterns of in situ methane oxidation by methane-oxidizing bacteria (methanotrophs), nature’s main biological mechanism for methane suppression, are often inconsistent with laboratory predictions. For example, one would expect a strong relationship between methanotroph ecology and Cu level because methanotrophs require Cu to sustain particulate methane monooxygenase (pMMO), the most efficient enzyme for methane oxidation. However, no correlation has been observed in nature, which is surprising because methane monooxygenase (MMO) gene expression has been unequivocally linked to Cu availability. Here we provide a fundamental explanation for this lack of correlation. We propose that MMO expression in nature is largely controlled by solid-phase Cu geochemistry and the relative ability of Cu acquisition systems in methanotrophs, such as methanobactins (mb), to obtain Cu from mineral sources. To test this hypothesis, RT-PCR expression assays were developed for Methylosinus trichosporium OB3b (which produces mb) to quantify pMMO, soluble MMO (the alternate MMO expressed when Cu is “unavailable”), and 16S-rRNA gene expression under progressively more stringent Cu supply conditions. When Cu was provided as CuCl2, pMMO transcript levels increased significantly consistent with laboratory work. However, when Cu was provided as Cu-doped iron oxide, pMMO transcript levels increased only when mb was also present. Finally, when Cu was provided as Cu-doped borosilicate glass, mb expression patterns varied depending on the ambient Cu supply ratio. Cu geochemistry clearly influences MMO expression in terrestrial systems, and, as such, local Cu mineralogy might provide an explanation for methane oxidation patterns in the natural environment.

methanotroph | bioweathering | methane oxidation | particulate methane monooxygenase | real-time RT-PCR

Methane-oxidizing bacteria (methanotrophs) are nature’s primary biological mechanism for reducing levels of atmospheric methane, the second most important greenhouse gas associated with global warming (1). However, habitat factors that influence methanotroph ecology and, implicitly, in situ methane oxidation rates are poorly understood despite extensive recent studies (2–5). Moisture content, pH, and oxygen, methane, and nitrogen levels have all been studied and conditionally shown to influence methanotrophic activity, but none of these factors provides a consistent explanation for the distribution of methane-oxidizing organisms in nature. Interestingly, copper (Cu), which is central to metabolism in methanotrophic bacteria (2), has not been studied in detail in situ, which is very surprising because Cu is an essential component of particulate methane monooxygenase (pMMO), the most efficient enzyme at methane catalysis. Furthermore, Cu regulates methane monooxygenase (MMO) expression in methanotrophs that express both pMMO and soluble MMO (sMMO); the alternate methane-oxidation enzyme in many organisms, and also affects the selection of type I versus type II strains in ecosystems (6–8). Therefore, one would expect Cu to be very important to methanotroph selection under any circumstances, and the question is why Cu has not been shown to be significant to methanotroph ecology beyond laboratory settings.

Here we provide a fundamental explanation for why in situ Cu conditions and associated methanotroph ecology often do not correlate in the natural environment, which is based on recently identified Cu-acquisition systems in methanotrophs that influence Cu availability. We propose that in situ methanotrophic ecology is controlled by the relative ability of some methanotrophs to mobilize and acquire Cu from mineral and organic solid phases to support pMMO expression. Specifically, a small, fluorescent chromopeptide, called methanobactin (mb) (9–11), was purified from Methylosinus trichosporium OB3b (a type II methanotroph that expresses both pMMO and sMMO), which mediates Cu acquisition and promotes pMMO expression in this organism. We suggest that Cu sequestration by mb from environmental Cu sources is the rate-limiting step in in situ pMMO expression, and, as such, mb:Cu interactions can explain where and when pMMO is expressed, methanotroph ecology, and possibly methane oxidation patterns in nature. As background, although only the mb from M. trichosporium OB3b has been purified, there is growing evidence that mb is not unique, with similar molecules being seen in Methylococcus capsulatus (Bath) and other species that express both pMMO and sMMO [see supporting information (SI) Text and SI Figs. 3 and 4]. As such, mb-like molecules may be quite common in nature and may play a previously unidentified role in the regulation of methanotrophic activity in geochemical settings.

To test this hypothesis, M. trichosporium OB3b was used as a model system to examine MMO expression patterns under different mb and Cu conditions. Three gene expression assays were developed by using real-time RT-PCR to quantify pMMO [pmoA, a β subunit of the pMMO (12, 13)], sMMO (mmoX, an α hydroxylase subunit of the sMMO), and 16S-rRNA (14) gene transcript levels to assess Cu acquisition (for MMO expression) under different Cu conditions. Specifically, Cu was provided to M. trichosporium OB3b cultures as CuCl2, synthetic Cu-doped
iron oxide, and Cu-doped borosilicate glass to simulate different soil and sedimentary environments, and gene transcript levels were quantified, with and without mb, over time. Expression patterns were then compared with Cu mb-sequestration patterns from three mineral sources to determine whether MMO expression paralleled mb-mediated Cu release from nonsynthetic minerals to verify that MMO expression might, in fact, be mediated by mb in mineral systems.

**Results**

**mb Sequestration of Cu from Different Soil and Mineral Sources.** A proof-of-concept experiment on two soils and one mineral was performed to assess how Cu solid-phase abundance and chemical speciation affected abiotic Cu release by mb. Three different proportional masses of mb were added to three sets of buffered, aquatic slurries containing two oxisols (Ox 1 and Ox 2) collected from Barro Colorado Island, Panama (see SI Text and SI Figs. 3 and 4), and anorthoclase (Na,KAlSi3O8), a primary silicate mineral, respectively. Table 1 shows that mb significantly increased Cu release from both oxisols but had little impact on Cu release from the silicate. Sequential extraction data (15) indicated that Cu in Ox 1 and Ox 2 was primarily associated with organics/oxides (50.5% and 64%, respectively), whereas anorthoclase Cu was largely framework-substituted (79% silicate-bound Cu by mass). Cu mineralogy and mb level clearly impact Cu release. As such, expression assays were performed using CuCl₂, synthetic Cu-doped iron oxide, and synthetic Cu-doped borosilicate glass (as defined sources) to assess whether gene expression paralleled predicted mb-mediated Cu release from these nonsynthetic sources.

**mb and MMO Expression from Defined Mineral Sources.** To examine how different mineral Cu sources affected gene expression, M. trichosporium OB3b cultures were pregrown in Cu-free media, subdivided into replicate 30-ml aliquots, and provided different combinations of Cu and mb. Fig. 1 summarizes transcript levels over time for *mmoX*, *pmoA*, and 16S-rRNA genes as a function of Cu source and mb level for all assays where total Cu supply was 0 or 5 μM. Either ANOVA (for comparing mb–Cu scenarios) or the *t* test (for comparing initial and final conditions within

<table>
<thead>
<tr>
<th>Mineral source</th>
<th>No mb</th>
<th>1:1 mb:Cu</th>
<th>5:1 mb:Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anorthoclase, 10 ppm</td>
<td>1.27±(0.76)</td>
<td>0.83 (0.16)</td>
<td>0.14 (1.01)</td>
</tr>
<tr>
<td>Oxisol 1, 103 ppm</td>
<td>0.71 (0.34)</td>
<td>6.99 (0.23)</td>
<td>11.8 (1.71)</td>
</tr>
<tr>
<td>Oxisol 2, 41 ppm</td>
<td>0.28 (0.91)</td>
<td>5.71 (0.34)</td>
<td>10.1 (0.24)</td>
</tr>
</tbody>
</table>

Data show Cu mass released into solution (μg/liter) with 95% confidence intervals provided in parentheses. Anorthoclase had Cu speciated as follows: 79% silicate, 6.2% organic and metal oxide, and 14.8% exchangeable and carbonate Cu fractions. Oxisol 1 and Oxisol 2 had only 40.5% and 30% silicate-bound Cu, respectively, with the majority of Cu present as organic/oxides (50.5% and 64%), ppm data show total solid-phase Cu levels in mineral sources. 1:1 and 5:1 are the mass ratios of total mb supplied to total Cu present in the mineral source in test vial.

![Fig. 1.](image-url)
scenarios) was used on log-transformed data to assess statistical significance among means in observed responses (with \( \alpha = 0.05 \) as the level of significance).

Fig. 1 A–C shows that when mb was provided alone to cultures pregrown in Cu-free media it had no effect on pmoA or 16S-rRNA transcript levels over 30 min (mb = 0 \( \mu \)M; \( t = 0.18 \), \( P = 0.87 \); mb = 1 \( \mu \)M; \( t = 1.80 \), \( P = 0.15 \); mb = 10 \( \mu \)M; \( t = 1.73 \), \( P = 0.16 \)), although mmoX transcript levels did increase slightly (mb = 0; \( t = 3.62 \), \( P = 0.04 \); mb = 1 \( \mu \)M; \( t = 3.63 \), \( P = 0.02 \); mb = 10 \( \mu \)M; \( t = 5.941 \), \( P < 0.01 \)). In contrast, Fig. 1 D–F indicates that pmoA transcript levels dramatically increased and mmoX transcript levels declined (albeit erratically) within 3 min after the addition of 5 \( \mu \)M Cu as CuCl2, although neither pmoA up-regulation nor mmoX repression, nor the level of 16S-rRNA gene transcript, differed significantly among different mb supply conditions (time = 30 min; pmoA, \( F_{2,27} = 0.94 \), \( P = 0.44 \); mmoX, \( F_{2,27} = 0.39 \), \( P = 0.69 \); 16S-rRNA, \( F_{2,27} = 0.82 \), \( P = 0.48 \)). These results are consistent with previous work where MMO “switchover” was regulated by ionic Cu (6, 8), although the lack of impact of mb on the rate of switchover is somewhat surprising. It had been expected that mb might accelerate Cu uptake (9, 10), similar to some siderophores with iron uptake (16); however, this was not seen within the detection limits of our methods.

In contrast to CuCl2, Fig. 1 G and J shows that when 5 \( \mu \)M Cu was provided as synthetic Cu-doped Fe oxide (80 ppm solid-phase Cu) or as Cu-doped borosilicate glass (80 ppm solid-phase Cu), the presence of mb significantly altered pmoA transcription patterns. For example, no significant change in pmoA transcript level was seen upon exposure to 5 \( \mu \)M Cu-oxide when no mb was provided (\( t = 2.36 \), \( P = 0.08 \)), whereas pmoA transcript levels increased significantly at both 0.2:1 and 2.0:1 mb:Cu supply ratios (all \( t > 12.5 \), \( P < 0.01 \)), especially for 2.0:1 mb:Cu. Fig. 1H shows that concurrent mmoX transcript levels declined only slightly when mb was not provided (\( t = 0.55 \), \( P = 0.61 \)), whereas mmoX expression was rapidly repressed (similar to when Cu was provided as CuCl2) when mb was also present (\( t > 6.61 \), \( P < 0.01 \)). Clearly, mb increases Cu availability for MMO switchover when Cu is provided as a solid-phase oxide, but, significantly, Cu was functionally not available over the time scale tested here when mb was not provided.

Finally, Fig. 1 J and K shows that pmoA transcript levels increased only at the 0.2:1 mb:Cu supply ratio when Cu was provided as a borosilicate glass (\( t = 12.5 \), \( P < 0.01 \)). Small increases in pmoA transcript levels were noted in the other glass treatments, but responses were much less pronounced (less than 1 order of magnitude; both \( t < 4.5 \), \( P = 0.01 \)). Furthermore, at the 2.0:1 mb:Cu supply ratio, pmoA transcript levels initially increased dramatically, but this response soon disappeared; we suspect that this resulted from initial release of surface Cu (mediated by mb) that was quickly quenched by excess mb that was also accumulating on the surface. Previous results showed that, when mb was oversupplied to a mineral surface (which we feel may be a laboratory artifact), mb tended to coat the surface of the mineral, hindering further Cu release (17). As such, slow, persistent pMMO expression seen in the 0.2:1 mb:Cu treatment likely results from reduced quenching of Cu release because there is less residual mb in the system.

Fig. 1 I and L further shows that 16S-rRNA transcript levels did not change when Cu was provided as either an oxide or silicate, suggesting that, although these two Cu forms do not necessarily trigger pMMO expression, they also do not cause detectable cell stress. Mb simply appears to make Cu more available from some insoluble sources, and, when Cu is present in a more refractory geochemical form, it neither positively nor negatively influences the cells. Overall, pmoA and mmoX expression patterns with the synthetic oxides and glasses were consistent with abiotic Cu release patterns for anorthoclase and the two Panamanian soils (Table 1), which implies that mb-mediated Cu release is a plausible explanation for what regulates MMO expression in a mineral environment.

**mb and Cu Toxicity Suppression.** To examine the ability of mb to reduce Cu toxicity in *M. trichosporium* OB3b, which is another suspected role for mb (9), the organism was exposed to increasing levels of Cu as CuCl2 and 16S-rRNA transcript levels were monitored over time. Initial 16S-rRNA levels (estimated before Cu addition) varied among treatments [Cu: 10(7.03) copies/ml, \( \pm 0.04 \) (95% confidence interval of log-transformed values); 10 \( \mu \)M: 10(19) copies/ml, \( \pm 0.04 \); 25 \( \mu \)M: 10(10) copies/ml, \( \pm 0.04 \)]; therefore, transcript levels were normalized to initial 16S-rRNA levels and reported as relative values. Fig. 2 shows 16S-rRNA transcript levels for 0, 10, and 25 \( \mu \)M Cu, and only when there was a 1:1 stoichiometric balance between mb and Cu in the media (i.e., Fig. 2B; 10 \( \mu \)M Cu and 10 \( \mu \)M mb) was 16S-rRNA gene transcription not immediately reduced by Cu supplementation. Interestingly, even in this case, 16S-rRNA transcript levels ultimately declined over 30 min, which suggests that an excess of mb relative to Cu might actually be needed to wholly protect the cells.

**Discussion**

Three extracellular roles have been proposed for mb relative to Cu availability, cell activity, and methanotroph ecology in natural systems. First, mb might act as a chelator for Cu to the cell, possibly supporting pMMO synthesis and activity. Second, mb might sequester scarcely available Cu from the cell’s growth environment (e.g., from insoluble mineral sources), allowing the cell to obtain Cu and express pMMO when Cu is less bioavailable. Finally, mb might play a role in cellular Cu defense; i.e., Cu is a toxic metal to almost all organisms (18), and mb–Cu binding might functionally "shield" Cu from the cell, reducing Cu toxicity. Our data support these three roles, except increased uptake rate with CuCl2, and suggest how mb might influence methanotroph ecology and probably activity in natural systems.
Since the first evidence of extracellular Cu-sequestering agents in methane-oxidizing bacteria (19), speculation has continued on what these compounds actually do for the cell. These compounds, originally called Cu-binding cofactor/compounds (cbc) (20, 21) or Cu-binding ligands (CBL) (22), were initially assumed to act as Cu transporters (maybe chaperones) that somehow inserted Cu into pMMO for function. However, evidence has since shown that mb (aka cbc or CBL) is not likely intrinsically associated with pMMO (13), although Choi et al. (10) have shown that the Cu–mb complex increases pMMO activity in cell-free and whole-cell preparations (relative to Cu or mb alone). Despite this observation, an environmental role for mb that is pertinent to these organisms in their natural habitat has not been established. Evidence indicates that mb can sequester non-Cu metals and also increases bioweathering rates (17, 23), but no previous work has shown how MMO gene expression is regulated by mineral Cu sources, which is essential for explaining methanotroph ecology and, implicitly, methane oxidation patterns within geologic systems.

Here we show that mb–Cu geochemistry directly affects MMO gene expression, which in turn implies that Cu sequestration by mb likely influences (maybe controls) methanotroph selection in nature. Two specific insights can be made from our data about mb alone. Despite this observation, an environmental role for mb that is pertinent to these organisms in their natural habitat has not been established. Evidence indicates that mb can sequester non-Cu metals and also increases bioweathering rates (17, 23), but no previous work has shown how MMO gene expression is regulated by mineral Cu sources, which is essential for explaining methanotroph ecology and, implicitly, methane oxidation patterns within geologic systems.

First, mb sequesters Cu and makes less available Cu more available, supporting pMMO expression in M. trichosporium OB3b. Given that mb is not unique, it not unreasonable to generalize this observation and suggest that mb-like molecules might broadly act as ligands that liberate Cu from refractory reservoirs, which allows internalization of Cu and activation of MMO in non-Cu media.

Whether mb–Cu directly regulates MMO expression by directly binding to the pMMO expression repressor (which then derepresses pMMO expression) or shuttles Cu to an intermediary molecule that acts on the repressor is not known (8). However, our data show that mb rapidly and unambiguously makes Cu available from the solid phase that, in turn, regulates MMO expression, potentially explaining MMO prevalence in the environment. Although this general observation is likely correct, one must be careful in how far it is extended to natural systems. For example, our work shows that mb mediates pMMO expression from mineral sources in M. trichosporium OB3b. However, nature is more diverse than the systems tested here; i.e., Cu minerals are less well defined (e.g., metalloorganic complexes), Cu mobility after release is more variable (e.g., soil vs. aquatic-mine sites), and/or other factors, such as methane or nitrogen levels, can affect MMO expression. Regardless, the results present a clear starting point for future field and other studies to verify our predictions in more natural settings.

The second insight indicates that mb reduces the cell toxicity to bioavailable Cu. This observation is consistent with results of Kim et al. (11) who showed that mb supplementation conditionally eliminated the “toxic” lag seen when these organisms are “shocked” with elevated Cu. Our new work extends Kim et al. (11) by showing that reductions in Cu toxicity are, at least in part, due to “protection” of the pMMO expression system. Specifically, Fig. 2 shows that mb temporarily shields the cells from Cu when provided in stoichiometric balance. Interestingly, the fact that elevated mineral Cu levels (without mb present) (Fig. 1) do not cause the same toxic effect suggests that Cu may not be that toxic to these organisms in nature because mineral Cu is often not readily available.

On an evolutionary level, it has been postulated that methanotrophs evolved their Cu-containing pMMO because methane is the most reduced electron donor in nature and a metal center with a high redox potential is needed to cleave C—H bonds in the methane molecule. As a result, methane oxidation requires Cu, which in turn, demands a strong Cu defense system. Hence, a molecular carrier for Cu, like mb, is logical because it might protect the cell both externally and internally from Cu toxicity. The significance of a toxicity-reducing, Cu-carrier molecule is especially relevant to methanotrophs given their “typical” habitat; i.e., geochemically distinct microaerophilic zones. In such locations, intense redox cycling leads to active precipitation of Mn and Fe oxides (24), and sequestration of metals via sorption, coprecipitation, and competitive organic complexation often dominate. Therefore, mb might be particularly critical for ecological success in such environments because mb allows the selective acquisition of Cu while also protecting the organisms against other potentially toxic metals.

Overall, this study provides strong evidence that mb mediates Cu release from the mineral phase, which alters Cu availability and allows pMMO gene expression in methanotrophs. As such, new work is strongly suggested that relates methanotroph habitat Cu geochemistry, in situ methanotroph selection, and actual methane oxidation rates. In theory, if one could calibrate MMO expression and methane oxidation rate within a relevant geochemical Cu setting, one might have a broad tool for predicting methanotroph activity potential from geochemical soil data. As an example, soil Cu analysis, including speciation, might be used to predict available Cu for MMO expression (and methane oxidation) analogous to Olsen “P” that is used in plant fertility studies (25). Although much work remains, this work is an important step in understanding MMO gene expression within the geologic setting of environmental methane oxidation and takes us one step closer to predicting better in situ methane flux rates, thus improving greenhouse gas models.

Materials and Methods

Sequential Extraction of Metals from Mineral Sources. Sequential extraction of the various minerals was performed to evaluate mobility and bioavailability of metals related to different soil components such as clay minerals, carbonate minerals, oxides, and soil organic matter (15). Major and trace elements were analyzed by using the JY138 Ultrace Inductively Coupled Plasma Atomic Emission Spectrometer (Jobin Yvon, Longjumeau, France) and the Elemental PlasmaQuad II+XS Inductively Coupled Plasma Mass Spectrometer (VG Biotech, Cheshire, U.K.), respectively. See SI Text for details of the procedures.

mb Production. mb was obtained for all assays from spent media from a 2.8-liter bioreactor (2.0-liter working volume) operated in continuous-culture mode from M. trichosphorium OB3b. The reactor feed was Cu-free NSM media (0.67 liter/day) and research-grade methane (99.99% purity; at 5-ml/min); all other reactor feed was Cu-free NSM media (0.67 liter/day) and research-grade methane (99.99% purity; at 5-ml/min); all other procedures were described previously (11). Typically, 90% of the reactor volume was harvested per collection event, which was centrifuged at 9,000 × g for 20 min, and filtered immediately (0.20-μm polycarbonate membrane filters; Pall Corp., East Hills, NY) to remove cells. The filtrate was then passed through a series of tC18-SepPak columns (Waters, Milford, MA) that had been prerinse with reagent-grade Milli-Q water, which were subsequently eluted with 60% acetonitrile. Resulting solutions were freeze-dried overnight (Freezone 4.5; Labconco, Kansas City, MO). Freeze-dried residues (from a series of harvesting events) were combined, homogenized, and stored under moisture-free conditions at ~20°C until enough mb was collected for the entire expression assay experimental program. Before each gene expression experiment, a fresh stock solution of mb was prepared from the stock by dissolving 10 mg of crude preparation mb into 1 ml of 4°C Cu-free NSM medium buffered at pH 7.0. Abundances of mb were verified by spectrophotometric analysis (21).

Abiotic Copper Sequestration Experiments with Soils. To assess whether mb sequestered Cu from selected nonsynthetic soils,
two soils and one silicate mineral were exposed to three levels of mb (Table 1). Blanks (with no soils added) were also included, thus creating a 4 × 3 treatment matrix in duplicate. For each assay, the soil or mineral was added at a level such that total Cu concentration (combined in solid and aqueous phases) equaled 7.9 μM per vial; mb mass was then added to stoichiometrically balance this level (i.e., 7.9 μM mb for 1:1 and 39.4 μM mb for 5:1). To initiate the assay, soil and mb were added to pre-acid-washed glass vials, containing 5 mM carbonate buffer (pH 8.0), and were gently agitated at 30 rpm (G24 Incubater Shaker, New Brunswick, Edison, NJ). After 24 h, 1–2 ml was collected, filtered (0.2-μm polycarbonate filter; Pall Corp.), and then acidified to pH < 2.0 with trace-metal quality nitric acid (Fisher Scientific, Waltham, MA). Samples were analyzed by using an Analyst 300 atomic adsorption unit with a HGA 850 graphite furnace (Fisher Scientific, Carlsbad, CA), and frozen on dry ice. Replicate samples were collected when time allowed. At the end of 30 min, the frozen samples were transferred to a −80°C freezer until further processing.

The same procedure was used in the second and third experiments; however, the second experiment examined the effect of different Cu sources on MMO and housekeeping (16S-rRNA) gene expression only. The second experiment compared gene expression in M. trichosporium OB3b when suddenly exposed to 5 μM total Cu (solid plus liquid phase) as CuCl₂, synthetic Cu-doped iron oxide, and synthetic Cu-doped borosilicate glass (24), respectively, and 0, 1, and 10 μM mb amendments (3 × 3 block design). The final experiment assessed expression responses to sudden exposures of 0, 10, or 25 μM CuCl₂ in the presence of 0, 1, and 10 μM mb amendments (a 3 × 3 block design) to assess cell responses to different levels of bioavailable Cu.

**Synthesis of Silicate Glasses and Iron Oxides.** Synthetic glasses and oxides were used in the gene expression assays (rather than natural Cu sources) because they were compositionally distinct and allowed greater control over solid-phase Cu level, although they were synthesized to simulate natural soils and sediments (26, 27). The Cu-doped borosilicate glasses were made from stock powders (wt % oxide: SiO₂ = 80.8, B₂O₃ = 12.0, Na₂O = 4.3, Al₂O₃ = 2.2) that were homogenized and melted in a graphite crucible at 950°C for 12 h. The resulting glass was crushed to a uniform sieve size of 125–250 μm in diameter, rinsed with reverse osmosis water, sonicated for 1 min under low power, and then air-dried. The Cu-doped ferrihydrite was prepared by using methods from Cornell and Schwertman (28). The precipitate was rinsed once with reverse osmosis water and centrifuged, placed in a dialysis bag, and submerged in reverse osmosis water for 48 h. The clean oxide product was freeze-dried before use.

**Culture Preparation for Expression Assays.** M. trichosporium OB3b stock cultures for each assay were pregrown from plate colonies in 125-ml Tygon-plugged serum vials (30-ml volume) under a 50% methane/50% air atmosphere at 30°C on an incubated orbital shaker table agitated at 200 rpm (G24 Incubater Shaker; New Brunswick). Once an OD₆₀₀ of 0.300 was achieved, sMMO activity was tested by using the o-dianisidine/ naphthalene spectrophotometric assay (21), and, if sMMO activity was high, the culture was centrifuged for 10 min at 10,000 × g at 4°C (Servall RC-58; Thermo Scientific, Waltham, MA). The resulting pellet was washed and centrifuged three more times to remove any extracellular mb (in fresh Cu-free NSM media), and the final pellet was resuspended in 30 ml of fresh media for each set of assays (see below). Before centrifugation, 1 ml of the stock culture had been transferred to a new 125-ml vial containing copper-free NSM liquid media, and a new stock culture was grown for the next expression assay.

**Gene Expression Experiments.** Three sets of expression experiments were performed. In the first, 10-ml aliquots of washed M. trichosporium OB3b culture were transferred (each) to three 30-ml crimp-sealed glass vials and amended with 5 ml of research-grade methane. The vials were agitated at 200 rpm (G24 Incubater Shaker; New Brunswick) at 30°C and allowed to equilibrate for 30 min. Each vial was then provided, respectively, no mb amendment, 5 μl of mb stock solution, and 50 μl of mb stock solution. Triplicate 300-μl samples were aseptically removed from the vials by using 1-ml TB syringes (time = 0 min samples), and additional samples were collected for 30 min. Each withdrawn volume was placed immediately into a microcentrifuge tube containing 1 ml of TRIzol LS reagent (Invitrogen, Carlsbad, CA), and frozen on dry ice. (Continued...)

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**Table 2. M. trichosporium OB3b primers and probes used in the study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>TaqMan probe (5′–3′)</th>
<th>Tₘ, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmoA*</td>
<td>TCTGGGGCTTGGACCTAYTTC</td>
<td>CCGACGACGACGAGGTGATG</td>
<td>[FAM]-CCACCTTGTGTCCTCCGCTCGCT-TAM</td>
<td>57</td>
</tr>
<tr>
<td>mmoX*</td>
<td>TCAACACCCGATCTTGCACAGC</td>
<td>TCCGGATCTCCGCCCCAACCTC</td>
<td>[FAM]-CCACCGGTTCCAGGCTTAC-TAM</td>
<td>56</td>
</tr>
<tr>
<td>16S‡</td>
<td>GGACAGCTTCCAGGTGTTCGAC</td>
<td>CTTGTGGGGAAGGAAAGTC</td>
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<td></td>
</tr>
</tbody>
</table>


Modified sequences from Gulludge et al. (29) and Holmes et al. (30).
and test toxicity effects because its expression is very sensitive to changing conditions (14). Primer sequences for the M. trichosporium 16S-rRNA were adapted from those presented by Gulledge et al. (29) and Holmes et al. (30). SYBR green was used to detect 16S-rRNA gene responses (the 16S-rRNA amplicons were too short to use TaqMan probes), whereas TaqMan probes were used for the MMO assays. Postanalysis melt curves were always used to verify quality in the SYBR green detection system.

Reverse transcription and real-time PCR were conducted by using the TaqMan EZ RT-PCR Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions, except reaction volumes were scaled to 25 µl with 15 pmol of primers and 6.25 pmol of probe (for mmoX and pmoA). The PCR program on iCycler (Bio-Rad, Hercules, CA) was 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, and 40 cycles at 94°C (20 sec), annealing temperature (Table 2) (60 sec), and 72°C (30 sec). Reaction standards were developed to monitor RT-PCRs; amplicons from each reaction (using iQ supermix PCR solution; Bio-Rad, Hercules, CA) were ligated into a pCR-TOPO vector and cloned into TOP10 chemically competent Escherichia coli (Invitrogen, Carlsbad, CA). Plasmid vectors were extracted by using a High Pure Plasmid Isolation Kit (Roche Diagnostics, Indianapolis, IN). RNA standard was synthesized from a reverse transcription reaction using the T7 RiboMAX Large-Scale Production System (Promega, Madison, WI) for calibration.

Data Analysis. Copy-number values were log-transformed to ensure normality before statistical analysis. Either ANOVA or t test determined significance among means with α = 0.05 (P < 0.05) as the level of significance. All samples for Cu analysis were performed in triplicate from which an average sample value was determined from each of the duplicate samples collected. Cu values were always background-corrected to account for slight Cu carryover from the media.

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