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Competition between Metals for Binding to Methanobactin Enables Expression of Soluble Methane Monooxygenase in the Presence of Copper

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It is well known that copper is a key factor regulating expression of the two forms of methane monooxygenase found in proteobacterial methanotrophs. Of these forms, the cytoplasmic, or soluble, methane monooxygenase (sMMO) is expressed only at low copper concentrations. The membrane-bound, or particulate, methane monooxygenase (pMMO) is constitutively expressed with respect to copper, and such expression increases with increasing copper. Recent findings have shown that copper uptake is mediated by a modified polypeptide, or chalkophore, termed methanobactin. Although methanobactin has high specificity for copper, it can bind other metals, e.g., gold. Here we show that in *Methylosinus trichosporium* OB3b, sMMO is expressed and active in the presence of copper if gold is also simultaneously present. Such expression appears to be due to gold binding to methanobactin produced by *M. trichosporium* OB3b, thereby limiting copper uptake. Such expression and activity, however, was significantly reduced if methanobactin preloaded with copper was also added. Further, quantitative reverse transcriptase PCR (RT-qPCR) of transcripts of genes encoding polypeptides of both forms of MMO and SDS-PAGE results indicate that both sMMO and pMMO can be expressed when copper and gold are present, as gold effectively competes with copper for binding to methanobactin. Such findings suggest that under certain geochemical conditions, both forms of MMO may be expressed and active *in situ*. Finally, these findings also suggest strategies whereby field sites can be manipulated to enhance sMMO expression, i.e., through the addition of a metal that can compete with copper for binding to methanobactin.

The increased availability of methane through industrial practices such as hydraulic fracturing has enhanced interest in using methanotrophs, prokaryotes that thrive on methane as their sole source of carbon and energy, to convert methane to more valuable products, e.g., liquid biofuels, plastics, and protein to supplement animal feed (1–7). Methanotrophs have also received increasing attention given that methane is a very potent greenhouse gas, with a global warming potential 28 to 34 times that of carbon dioxide over a 100-year period (8), and methanotrophs are estimated to remove up to 90% of the methane produced in anaerobic soils (9). Methanotrophs are widespread in the environment, found in diverse locations such as landfill cover, forest, agricultural, and volcanic soils; freshwater and marine sediments; and sewage sludge (6, 10, 11). Many are amenable to genetic manipulation (12–18).

An important issue, however, in the use of methanotrophy for any purpose is that the first step in methane oxidation, the conversion of methane to methanol, is performed by two different forms of methane monooxygenase (MMO) with different oxidation kinetics and affinities for methane. One form, the soluble methane monooxygenase (sMMO), is found in the cytoplasm and has relatively high turnover (maximal whole-cell methane oxidation rate [V_{max}] of ~730 nmol · min^{-1} · mg of protein^{-1}) but poor affinity (whole-cell binding constant [K_s] of ~90 μM). The other form, the particulate methane monooxygenase (pMMO), is found in the intracytoplasmic membranes and has relatively low turnover (V_{max}, ~80 nmol · min^{-1} · mg of protein^{-1}) but greater affinity (K_s, ~8 μM) (19). The form of MMO expressed is of importance, as the utility of methanotrophs for various applications, including methane oxidation and pollutant degradation, is strongly dependent on which form of MMO is expressed (19, 20).

It is well known that copper plays a key role in the expression of the two forms of MMO. sMMO expression is evident only when copper concentrations in the growth medium are low (e.g., submicromolar). pMMO is constitutively expressed, however, with respect to copper, and its expression increases with increasing copper (21, 22). For the sequestration of copper, many methanotrophs have been found to synthesize different chalkophores for copper uptake, akin to siderophores used for iron uptake. These chalkophores, collectively called methanobactin, are small modified polypeptides (<1,200 Da) with two heterocyclic rings (one of which is an oxazolone ring and the other of which is either an oxazolone, imidazolone, or pyrazinedione ring) and associated enethiol groups (23–26). Copper is bound by methanobactin with very high affinity (reported affinities range from 10^{20} to 10^{34} M^{-1} [24, 27–30]) using nitrogen from both heterocyclic rings and the sulfur of the enethiol groups.

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The metal binding moieties of methanobactin, however, can bind many other metals, but generally with much lower affinities, $\sim 10^5$ to 10^7 M^{-1} (30). Detailed study of methanobactin from *Methylosinus trichosporium* OB3b indicated that these metals can be divided into two general groups. The first group, group A, are those metals that are bound to both heterocyclic rings of methanobactin (both being oxazolone rings) and are reduced following binding. These metals include Cu(II), Ag(I), Au(III), Hg(II), and Pb(II). The second group, group B, includes those metals that are bound to only one oxazolone ring and are not reduced after binding. These metals include Cd(II), Co(II), Fe(III), Mn(II), Ni(II), and Zn(II) (30).

Further, it has been found that at least one metal, Hg(II), binds to methanobactin with rapid kinetics, with most binding occurring in the dead time of the stopped-flow system used (1.8 ms). For the remainder, binding was observed to have a rate of 640 s^{-1} (31). Therefore, despite the presence of copper, mercury can be irreversibly bound to methanobactin. It may be that in the presence of copper, sMMO expression may be possible due to the inability of methanotrophs to collect copper via methanobactin because of the presence of competing metals. Here we report on the effects of various group A and B metals added in conjunction with copper on expression of sMMO and pMMO in *M. trichosporium* OB3b.

TABLE 1 Growth and sMMO activity of *M. trichosporium* OB3b in the presence of 2 μM copper and various concentrations of competing metals for binding to methanobactin

Competing metal(s)	Metal concn(s) (μM)	Growth of *M. trichosporium* OB3b[a]	sMMO activity[b]
None (2 μM copper only)		Yes	No
Group A			
Hg	5	No	ND
Ag	5	No	ND
Au	5	Yes	Yes
Group B			
Zn	10	Yes	No
Mn	10	Yes	No
Mo	10	Yes	No
Ni	10	Yes	No
Co	10	Yes	No
Zn + Ni + Mn + Mo + Co	25, 50, 100, 250, and 500	Yes	No

[a] Positive growth defined as OD_{600} of >0.2 within 3 days from an initial OD_{600} of ~0.05.
[b] sMMO activity determined using the naphthalene assay (35). ND, not determined due to lack of growth.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *M. trichosporium* OB3b was grown on nitrate mineral salt (NMS) medium (32) at 30°C in 250-ml sidearm Erlenmeyer flasks shaken at 200 rpm in the dark either with no added copper (creating a background copper concentration of 0.03 ± 0.01 μM) or with 2 μM copper (added as $CuCl_2$). For consideration of the effect of competing metals for binding to methanobactin, various concentrations of Ag as $AgNO_3$, Hg as $HgCl_2$, Au as $HAuCl_4$, Zn as $ZnSO_4 \cdot 7H_2O$, Mn as $MnCl_2 \cdot 4H_2O$, Mo as $Na_2MoO_4 \cdot 2H_2O$, Ni as $NiCl_2 \cdot 6H_2O$, and Co as $CoCl_2 \cdot 6H_2O$ were added from metal stock solutions of either 10 mM or 100 mM. All chemicals were of American Chemical Society grade or better. Specific concentrations and combinations of metals considered are shown in Table 1. Methanobactin from *M. trichosporium* OB3b was purified as described previously (33). Copper-methanobactin was then prepared by adding equimolar amounts of copper and methanobactin to create a 5 mM stock solution. Copper-methanobactin was freshly prepared at 30°C under constant mixing at 200 rpm in the dark for 1 h before use. Copper-methanobactin was then added to some cultures at a concentration of 5 μM. All conditions were run using biological duplicates.

Protein measurements. The procedure outlined by Semrau et al. (34) was used to quantify protein concentrations. Briefly, protein was measured using the Bradford assay (Bio-Rad Laboratories) after concentration of 5 ml of the culture to 1 ml and digestion in 2 M NaOH (0.4 ml 5 M NaOH per 1.0 ml of culture) at 98°C for 15 min. A plot of protein concentrations of cultures of *M. trichosporium* OB3b cells at different optical densities at 600 nm (OD_{600}) yielded a linear regression of an OD_{595} value of 1.0, equal to 850 μg of protein per ml, with a coefficient of determination value (R^2) of 0.995. This correlation was used to calculate protein concentration for all cultures.

Metal measurements. Cultures were centrifuged at 5,000 × *g* for 10 min at 4°C. Supernatant samples were stored at −80°C and cell pellets resuspended in 1 ml of fresh NMS medium before storage at −80°C. Supernatant samples were then diluted in NMS medium with 5% (vol/vol) HNO_3 to achieve a final concentration of 2% (vol/vol) HNO_3. Cell suspensions were acidified in 1 ml of 70% (vol/vol) HNO_3 and incubated for 2 h at 95°C (31). The acidified cell suspensions were mixed by inverting the tubes every 30 min. Copper and gold associated with biomass and supernatant were analyzed using an inductively coupled plasma mass spectrometry (ICP-MS) instrument (PerkinElmer, Waltham, MA). Duplicate biological samples were used for each combination of copper, gold, and copper-methanobactin, with each replicate measured five times.

Measurement of sMMO activity. The activity of soluble methane monooxygenase (sMMO) was also monitored by performing a colorimetric assay as developed by Brusseau et al. (35). Briefly, a 2-ml aliquot of *M. trichosporium* OB3b under each growth condition was transferred to a 2-ml Eppendorf centrifuge tube and naphthalene was added. The cultures were then incubated for 1 h at 30°C and shaken at 200 rpm. Cells were pelleted by centrifugation for 5 min at 5,800 × *g*. Tetrazotized *o*-dianisidine (130 μl of 4.21 mM) was added to 1.3 ml of supernatant in a 1.5-ml cuvette. All experiments were performed using biological duplicates.

Nucleic acid extraction and real-time quantitative reverse transcriptase PCR (RT-qPCR). RNA was extracted from *M. trichosporium* OB3b grown in 250-ml sidearm flasks as described previously (31, 34). Briefly, 2.5 ml of stop solution (5% buffer equilibrated phenol [pH 7.3] in ethanol) was added to individual cultures (22.5 ml each) to stop any new mRNA synthesis. Cell pellets were then collected by centrifugation at 5,000 × *g* for 10 min at 4°C. The cell pellet was then resuspended in 0.75 ml of extraction buffer, and subsequent steps were performed to extract RNA as described previously (31, 34). RNA was then subjected to RNase-free DNase treatment until free of DNA contamination. RNA was checked for any DNA contamination via PCR amplification of the 16S rRNA gene, and the concentration of purified RNA was determined spectrophotometrically (NanoDrop ND1000; NanoDrop Technologies, Inc., Wilmington, DE). RNA samples were stored at −80°C and used for cDNA synthesis within 2 days of extraction. cDNA was prepared from DNA-free RNA samples (500 ng each) by reverse transcription using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Relative expression of the *pmoA*, *mmoX*, and *mbnA* genes in *M. trichosporium* OB3b grown at various concentrations of copper, gold, and/or copper-methanobactin was quantified by RT-qPCR. Amplifications of *pmoA*, *mmoX*, and *mbnA* and 16S rRNA genes were performed using primer pairs (5′-3′) TTCTGGGGCTGGACCTAYTTC and CCGACAGC AGCAGGATGATG for *pmoA* (amplicon length, 94 bp), TCAACACCGA TCTSAACAACG and TCCAGATTCCRCCCCAATCC for *mmoX* (amplicon length, 153 bp), TGGAAACTCCCTTAGGAGGAA and CTGCAC

GGATAGCACGAAC for *mbnA* (amplicon length, 107 bp), and GCAGA ACCTTACCAGCTTTTGAC and CCCTTGCGGGAAGGAAGTC for 16S rRNA (amplicon length, 66 bp) (34). The threshold cycle (C_T) values were measured from cDNA preparations in 96-well PCR plates using the Mx3000P qPCR system (Stratagene, La Jolla, CA). Each RT-qPCR was carried out in a final volume of 20 μl containing 0.8 μl of cDNA, 1× Brilliant III SYBR green qPCR Mastermix (Agilent Technologies, Santa Clara, CA), 15 nM ROX dye, a 0.5 μM concentration each of gene-specific forward and reverse primers, and nuclease-free sterile water (Ambion, Life Technologies, Grand Island, NY). A three-step PCR program, with an initial denaturation at 95°C for 10 min and 40 cycles of denaturation (95°C for 30 s), annealing (58°C for 20 s), and extension (68°C for 30 s), was performed. The specificities of qPCR products were verified by melting curve, gel electrophoresis, and sequencing. Average C_T values obtained from MxPro software (Stratagene, La Jolla, CA) were always in the linear range of amplification as determined by the standard curve for each gene (see Fig. S1 in the supplemental material). These C_T values were then used to calculate the relative gene expression levels with 16S rRNA as the housekeeping gene by the comparative threshold amplification cycle method ($2^{-\Delta\Delta CT}$), as described previously (36). Measurements were performed for two biological replicates for each growth condition.

Isolation of soluble and membrane fractions. *M. trichosporium* OB3b was also cultured for soluble and membrane fractions. Cells from NMS agar plates were used to inoculate four 250-ml Erlenmeyer flasks each with 50 ml of NMS medium with no added copper (i.e., a background concentration of 0.03 ± 0.01 μM copper) at 30°C. After an OD_{600} of ~0.7 was reached, these cultures were used to inoculate four 2-liter Erlenmeyer flasks each with 250 ml of NMS medium with no added copper. After these cultures subsequently grew to an OD_{600} of ~0.7, this combined 1 liter of cell culture was used to inoculate a 14-liter BioFlo and Celligen 310 fermentor (New Brunswick, Enfield, CT), again with no added copper. Growth was promoted by providing continuous methane-air feed rates of 70 ml min^{-1} for methane and 700 ml min^{-1} for air. After 24 h, the fermentor was filled with 8 liters of NMS medium, again with no added copper, and incubated for 24 h. Following the incubation period, 7 liters of culture was removed. The copper concentration in the remaining medium in the fermentor was then increased to 2 μM as $CuCl_2$, and an additional 7 liters of NMS medium amended with 2 μM copper as $CuCl_2$ was added. The culture was incubated for 24 h, and 7 liters of culture was then removed. The remaining medium was then amended with 5 μM Au as $HAuCl_4$, and the fermentor was filled with an additional 7 liters of NMS medium containing 2 μM copper as $CuCl_2$ plus 5 μM Au as $HAuCl_4$. After another 24 h, 7 liters of culture was removed. The remaining medium was then amended with 5 μM copper-methanobactin, and the fermentor was filled with an additional 7 liters of NMS medium containing of 2 μM copper as $CuCl_2$, 5 μM Au as $HAuCl_4$, and 5 μM copper-methanobactin. After 24 h, the entire fermentor culture was harvested. Cells from the fermentor were collected by centrifugation at 4,550 × *g* for 30 min, and the cells were resuspended in minimal volume of 10 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.3). The cell suspension was then centrifuged at 14,600 × *g* for 15 min and the pellet resuspended in 30 mM MOPS buffer (pH 7.3) to a final volume of 150 ml. The washed cell suspension was deoxygenated by 4 cycles of vacuum, followed by purging with argon. All manipulations after this were conducted in a type B Coy anaerobic chamber (95% argon and 5% hydrogen). Cells and subsequent fractions were kept at 4°C. The deoxygenated cell suspension was lysed with four passes on an Emulsiflex high-pressure homogenizer (Avestin, Ottawa, ON, Canada) at 25,000 to 30,000 lb · in^{-2}. The lysate was centrifuged at 14,600 × *g* for 15 min. The pellet was discarded, and the supernatant was centrifuged at 244,000 × *g* for 1.5 h. The supernatant was centrifuged a second time at 244,000 × *g* for 1.5 h to remove contaminating membranes, and the supernatant fraction was regarded as the soluble fraction. This supernatant was divided into smaller fractions and stored at −20°C. The pellets from the centrifugations at 244,000 × *g* were resuspended using a Dounce homogenizer in 30 mM MOPS and 1 M KCl buffer (pH 7.3) and centrifuged at 244,000 × *g* for 1.5 h. The membrane pellet was resuspended in a minimal volume of 30 mM MOPS buffer (pH 7.3) and stored at −20°C.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on precast 12% bis-tris gels with MOPS-SDS running buffer as specified by the manufacturer (Invitrogen, NY). The gels were run at 60 V for 30 min and then at 100 V for another 3 h. Approximately 15-μg quantities of total protein from soluble and membrane fractions were loaded in the gels together with prestained broad-range SDS-PAGE standards (Bio-Rad, CA). Finally, the gels were stained with Coomassie brilliant blue R.

Prediction of metal speciation. Copper speciation in NMS medium amended with either copper or copper and gold was predicted using Visual Minteq version 3.0 (http://vminteq.lwr.kth.se/) using the assumption of equilibrium. pH and temperature were set at 6.8 and 30°C, respectively, to simulate growth conditions. The model parameter and interface model were set to the default hydrous ferric oxide model (37) and a 2-site protonation model (38), respectively.

Mixed-metal binding by methanobactin from *M. trichosporium* OB3b. Binding of gold and copper by methanobactin under mixed-metal conditions was determined in solutions containing $CuCl_2$, $HAuCl_4$, and methanobactin in molar ratios of 0.25:0.25:1, 0.5:0.5:1, 1:1:1, 1.5:1.5:1, and 2:2:1 as previously described for copper-mercury-methanobactin mixtures (39). Briefly, 100 μM methanobactin was added to various concentrations of gold and copper with a total volume of 20 ml and incubated with stirring (200 rpm) at room temperature for 5 min. Following this incubation period, the solution was loaded onto preequilibrated Sep-Pak cartridges (Millipore Corporation, Billerica, MA). Sep-Pak cartridges were equilibrated by one wash with 3 ml of methanol, one wash of 3 ml of acetonitrile, one wash of 3 ml of methanol, and three washes of 3 ml of >18-MΩ · cm of H_2O. Once the reaction solution was loaded, the Sep-Pak cartridges were washed three times with 6 ml of >18 MΩ · cm H_2O and the methanobactin fraction was eluted with 60% acetonitrile–40% H_2O >18-MΩ · cm H_2O. Both the wash and eluted sample were diluted with a 5% trace-metal-grade HCl–5% trace-metal-grade HNO_3–90% >18-MΩ · cm H_2O solution. Copper and gold concentrations in the wash solution and 60% acetonitrile eluent were determined on an Agilent 55 AA atomic absorption spectrometer (Agilent Technologies, Santa Clara, CA) run in flame mode. All measurements were done for triplicate samples, and each sample was measured five times.

Gold displacement of copper bound to methanobactin. Displacement of copper prebound to methanobactin was determined by monitoring changes in UV-visible (UV-Vis) absorption spectra following the addition of an equimolar concentration of gold as $HAuCl_4$. Briefly, a methanobactin concentration of 75 μM was incubated with an equimolar amount of copper as $CuCl_2$ as described above. Gold was then added at an equimolar amount as $HAuCl_4$, and the UV-Vis absorption spectra were measured immediately afterwards.

The kinetics for gold displacement of copper from methanobactin were determined by measuring absorption changes at 336 nm, using a four-syringe Biologic SFM/4000/S stopped-flow reactor coupled to a MOS-500 spectrophotometer (Biologic Science Instrument SA, Claix, France) at room temperature (23 to 24°C). Metal stock solutions of $CuCl_2$ or $HAuCl_4$ were prepared in >18 MΩ · cm of H_2O, followed by the addition of Cu(II) to methanobactin in a molar ratio of Cu(II) to methanobactin of 1 to 1. The stock solutions for methanobactin were prepared by dissolving freeze-dried methanobactin in >18 MΩ · cm of H_2O. The stock solutions of $CuCl_2$, $HAuCl_4$, and copper-methanobactin were chilled on ice and then filtered through a 0.22-μm filter before being loaded into sample syringes. The final concentration of the stock copper-methanobactin solution after filtration was determined by UV-visible absorption spectroscopy (33). The path length for the cuvette used in the Biologic SFM/4000/S stopped-flow reactor was 1.5 mm. The dead time of the system was 1.4 ms. The reaction mixture contained 400 μM copper-methanobactin and 400 μM $HAuCl_4$. Rates obtained were an average of 5

traces, and the experiment was repeated four times. The rates were determined by fitting the traces to the exponential function in Bio-Kine operational software (Biologic Science Instrument SA).

Statistical analyses. Data were analyzed using unpaired, two-tailed Student's *t* tests assuming equal variance between groups to determine any significant differences in the response of *M. trichosporium* OB3b to different culture conditions.

RESULTS

Growth and expression of sMMO in the presence of copper and various metals. An initial survey of both group A and B metals was performed to determine if, in the increased presence of these metals, sMMO expression was possible in the simultaneous presence of 2 μM copper. As shown in Table 1, *M. trichosporium* OB3b grew in the presence of many group B metals (Zn, Mn, Mo, Ni, and Co), either singly or in combination at concentrations as high as 500 μM each. In no case, however, was any sMMO activity observed via the naphthalene assay. Given these results, further study of the effect of these metals on methanotrophic activity was not pursued. Of the three tested group A metals, mercury and silver were toxic at a concentration as low as 5 μM (i.e., no growth was observed) and were also not considered further. Growth, however, occurred in the presence of 5 μM gold, and despite the presence of 2 μM copper, sMMO activity was observed via the naphthalene assay. The impact of gold on methanotrophic activity and gene expression was thus explored in more detail.

***pmoA*, *mmoX*, and *mbnA* expression in *M. trichosporium* OB3b in the presence of copper, gold, and copper-methanobactin.** Figure 1A shows that *pmoA* expression was constitutive in the presence of 2 μM copper regardless of the simultaneous presence of various amounts gold and copper-methanobactin, and such expression was not significantly different across all tested conditions ($P > 0.05$). As expected, in the presence of 2 μM copper, very little *mmoX* expression was observed (Fig. 1B). In the presence of 2 μM copper and 5 μM gold, however, *mmoX* expression increased over 10,000-fold (significant at a *P* value of <0.003). In the presence of 2 μM copper, 5 μM gold, and 5 μM copper-methanobactin, very little *mmoX* expression was observed, and it was not significantly different from that observed in the presence of only 2 μM copper ($P < 0.4$). Interestingly, *mmoX* expression was ~5-fold greater in the presence of 2 μM copper and 5 μM gold than when the two metals were not added ($P < 0.007$). Expression of *mbnA* also varied in response to changing growth conditions, as shown in Fig. 1C. Specifically, *mbnA* expression increased significantly in the presence of 2 μM copper and 5 μM gold compared to that in the presence of 2 μM copper alone ($P < 0.03$) and was similar to that observed when neither copper nor gold was added ($P = 0.09$). When 5 μM copper-methanobactin was also added, expression of *mbnA* decreased but was not significantly different from the level of expression when *M. trichosporium* OB3b was grown either with 2 μM copper or with 2 μM copper and 5 μM gold ($P = 0.5$ and 0.12, respectively).

SDS-PAGE analyses of pMMO and sMMO polypeptides. To determine if sMMO polypeptides were formed in addition to gene expression, SDS-PAGE analyses were performed for both membrane and soluble fractions of *M. trichosporium* OB3b grown at various copper, gold, and copper-methanobactin concentrations. As shown in Fig. S2 in the supplemental material, it appears that polypeptides associated with sMMO and pMMO were present under all conditions but that sMMO expression was greatest in the absence of copper, gold, and copper-methanobactin and was lowest in the presence of 2 μM copper. When 5 μM gold was added in conjunction with 2 μM copper, expression of sMMO polypeptides appeared to increase, while pMMO polypeptides appeared to decrease. Finally, in the presence of 2 μM copper, 5 μM gold, and 5 μM copper-methanobactin, expression of sMMO polypeptides appeared to decrease compared to expression when *M. trichosporium* OB3b was grown in the presence of 2 μM copper and 5 μM gold, while pMMO polypeptides appeared to increase.

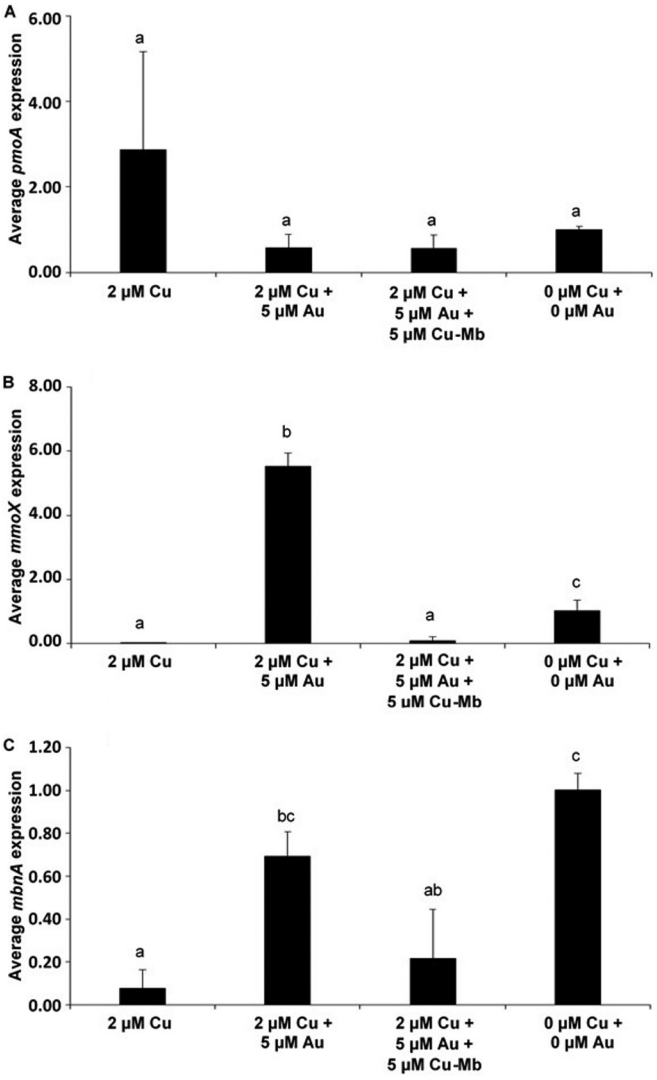

FIG 1 RT-qPCR of *pmoA* (A), *mmoX* (B), and *mbnA* (C) in *M. trichosporium* OB3b grown in the presence of copper (Cu), gold (Au), and copper-methanobactin (Cu-mb). Bars within each plot labeled by different letters are significantly different ($P < 0.05$).

sMMO activity in the presence of copper, gold, and copper-methanobactin. To determine if sMMO was active as well as its genes expressed in the presence of gold, copper, and methanobactin preequilibrated with copper, the naphthalene assay was used to monitor sMMO activity (35). As shown in Fig. 2, in the presence of 2 μM copper, no sMMO activity was observed. Clear evidence of naphthalene oxidation, however, was observed in cultures grown with no added copper, gold, or copper-methanobactin, as well as

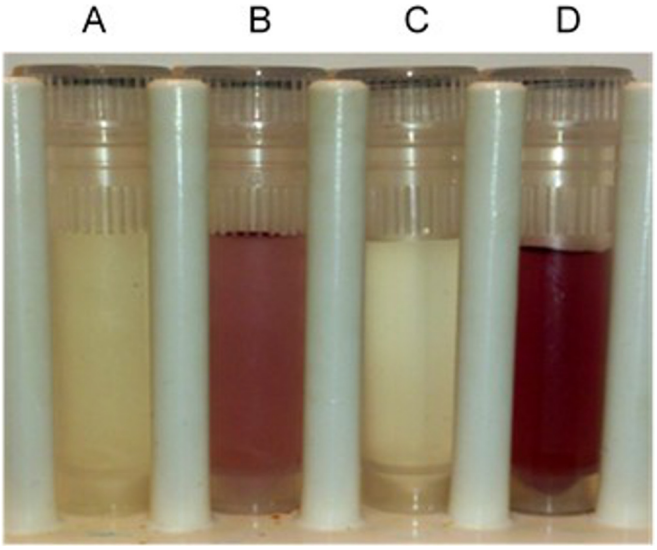

FIG 2 sMMO oxidation of naphthalene in *M. trichosporium* OB3b grown in the presence of various amounts of copper, gold, and copper-methanobactin, as follows: 2 μM copper (A); 2 μM copper and 5 μM gold (B); 2 μM copper, 5 μM gold, and 5 μM copper-methanobactin (C); and no added copper, gold, or copper-methanobactin (D).

in the presence of both 2 μM copper and 5 μM gold, indicating that the genes for sMMO were expressed and active enzyme was produced under these conditions. When 5 μM copper-methanobactin was added in addition to 2 μM copper and 5 μM gold, no sMMO activity was observed, and this is in agreement with the results of RT-qPCR and SDS-PAGE analyses.

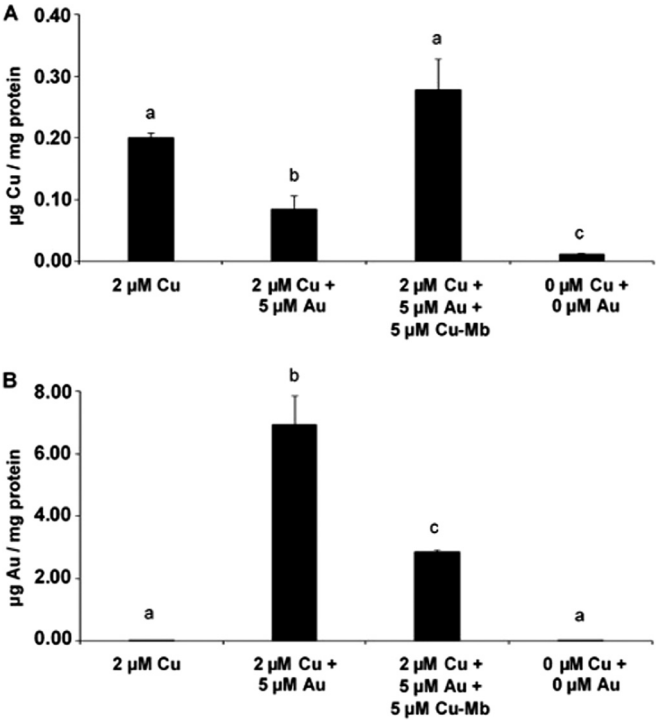

FIG 3 Metals associated with the biomass of *M. trichosporium* OB3b grown in the presence of copper (Cu), gold (Au), and copper-methanobactin. (A) Copper. (B) Gold. Bars in each plot labeled by different letters are significantly different ($P < 0.05$).

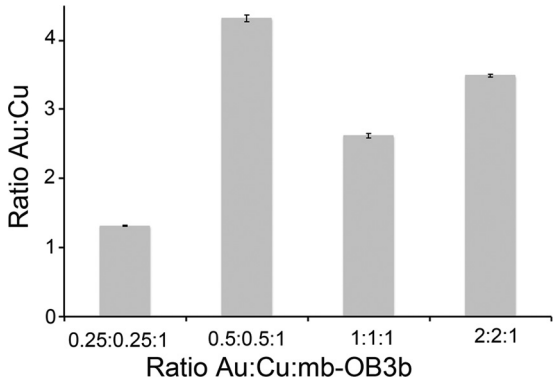

FIG 4 Ratio of gold to copper bound to methanobactin following incubation of Cu(II), Au(III), and methanobactin in molar ratios of 0.25:0.25:1, 0.5:0.5:1, 1:1:1, 1.5:1.5:1, and 2:2:1. Error bars represent the standard deviations of triplicate samples each measured five times.

Copper and gold associated with biomass. As shown in Fig. 3, ICP-MS analyses of the biomass of *M. trichosporium* OB3b indicated that in the presence of gold, the amount of copper associated with biomass significantly decreased, ~2.3-fold ($P < 0.03$). When copper was also added as copper-methanobactin, copper per unit biomass increased ~40% compared to that in cultures grown only in the presence of copper, but such an increase was not statistically significant ($P < 0.2$). Approximately 85 times more gold was associated with biomass than copper when both were present in the growth medium (~27 times more on a molar basis). In the simultaneous presence of 5 μM copper-methanobactin, the amount of gold per unit biomass was reduced by ~60% (significant at a P value of <0.03), but approximately 10 times more gold was associated with biomass than copper under these conditions (~3 times more on a molar basis).

Metal speciation. The MINTEQ program was run for two conditions: (i) the presence of 2 μM copper and (ii) the presence of 2 μM copper and 5 μM gold. The results (see Table S1 in the supplemental material) show no differences in the distribution of Cu^{2+} between these conditions, indicating that the observed response of *M. trichosporium* OB3b in the presence of gold and copper versus copper alone was not due to the addition of gold causing copper speciation to change.

Mixed-metal binding and gold displacement of copper from copper-methanobactin. To estimate the extent of competition between copper and gold for binding to methanobactin from *M. trichosporium* OB3b, copper, gold, and methanobactin were incubated in the presence of both metals at different molar ratios of gold to copper to methanobactin. Surprisingly, methanobactin preferentially bound Au(III) over Cu(II) at all metal-to-methanobactin ratios tested (Fig. 4). Based on these results, the capacity of Au(III) to displace copper already bound to methanobactin was examined. The UV-visible absorption spectral changes following the addition of Au(III) to copper-methanobactin were consistent with the displacement of copper (Fig. 5A). To determine the displacement rate, the decrease in absorbance at 336 nm was measured (Fig. 5B). The displacement showed an initial high rate, 67.89 ± 0.12 s^{-1}, followed by a low rate, 23.19 ± 0.014 s^{-1}, after 0.084 s.

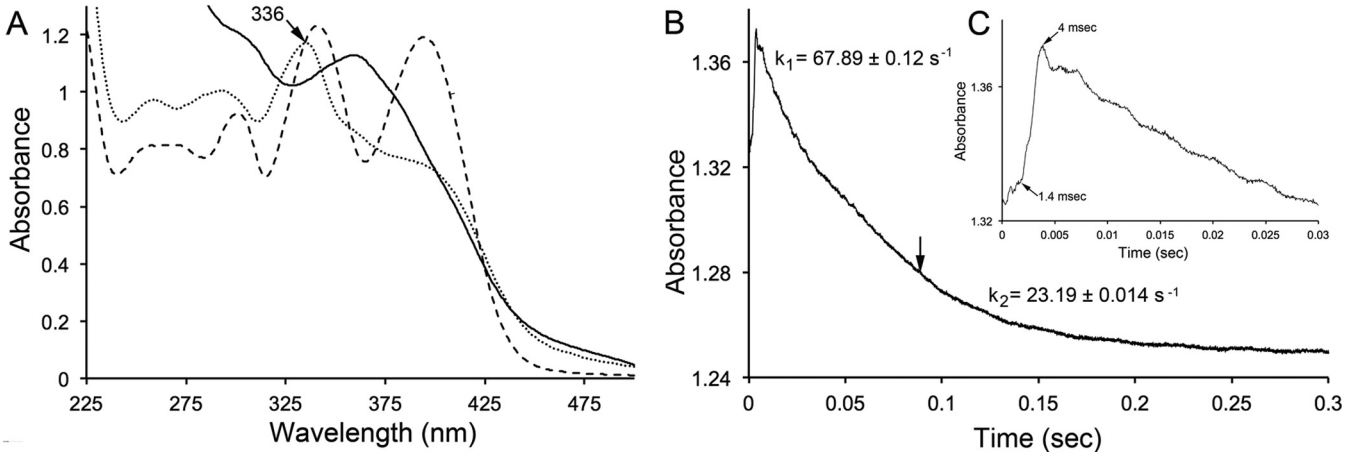

FIG 5 (A) UV-visible absorption spectra of copper-free methanobactin (dashed line), copper-methanobactin at a molar ratio of 1:1 (dotted line), and following the addition of an equimolar concentration of gold to copper-methanobactin (solid line). (B and C) Kinetics of copper displacement from copper-methanobactin by the addition of an equimolar concentration of gold. Absorbance changes were monitored at 336 nm following the addition of gold. The kinetics of copper displacement was biphasic, and the arrow indicates the transition from the initial high rate (k_1) to a secondary lower rate (k_2).

DISCUSSION

It is commonly stated that for those methanotrophs that can express both forms of MMO (i.e., the "switchover" strains), sMMO is expressed only when copper concentrations are low. Such a conclusion is based on laboratory experiments that used simple culture conditions to identify the response of methanotrophs to various amounts of copper (for examples, see references 21, 22, and 40). Although these experiments provided insight into the role of copper in methanotrophic physiology, one should keep in mind that natural environments are intrinsically much more complex, with the geochemistry of many environments poorly understood. It should also be stressed that there are very few reported examples of sMMO expression in nature (for examples, see references 41 to 43), raising the question, "Under what conditions do methanotrophs express sMMO *in situ*?"

To answer this question, one must consider not only the geochemistry of various natural environments, i.e., whether copper is present or not, but also the mechanism(s) by which methanotrophs collect copper, i.e., methanobactin. This chalkophore is well known to have a very high affinity for copper ($>10^{20}$ M^{-1}), but it has also been recently found that methanobactin from *M. trichosporium* OB3b can bind other metals (e.g., group A metals such as mercury and gold) and that copper cannot displace these after they are bound to methanobactin (30). Further, at least for mercury, binding to methanobactin is very rapid, and as a result, substantial mercury binding can occur even in the presence of copper (31).

With this in mind, we chose to rephrase the question above to instead ask, "What happens to copper uptake and resulting MMO expression in methanotrophs when other metals are present along with copper?" From the data presented here, it is obvious that group B metals (those that bind to only one of the oxazolone rings of methanobactin from *M. trichosporium* OB3b and are displaced if copper is subsequently added) have no effect on expression of sMMO in *M. trichosporium* OB3b. Although it was not measured, it appears that the presence of these metals had little impact on the ability of *M. trichosporium* OB3b to sequester copper (based on the lack of sMMO expression when these metals were added in excess). However, at least one group A metal, gold, can limit copper uptake by *M. trichosporium* OB3b. It appears that binding of gold by both oxazolone rings of methanobactin of *M. trichosporium* OB3b prevents copper from displacing gold once bound and, as a result, allows for sMMO expression and activity in the presence of copper. Further, substantially more gold than copper was found to be associated with biomass when both were added (Fig. 3).

Such a finding can be explained by considering the mixed-metal binding studies. It was found that in the presence of equimolar amounts of copper, gold, and methanobactin, more gold was associated with methanobactin than copper (Fig. 4). This is surprising, as the affinity of methanobactin for gold is reported to be many orders of magnitude lower than that measured for copper (30). It appears, as was found for mercury (31), that the kinetics of gold binding are rapid (Fig. 5) and possibly irreversible, and this may allow for the binding of gold in the presence of copper.

Thus, it should be stressed that it is inaccurate to predict, based on measurement of copper concentration alone, if sMMO will be expressed. Rather, it is expressed when methanotrophs are limited in their ability to sequester copper, e.g., through competition between metals for binding to methanobactin. This is supported by the finding that if methanobactin was preloaded with copper and added at a concentration of 5 μM, along with 2 μM copper and 5 μM gold, *mmoX* expression decreased significantly compared to expression in the presence of 2 μM copper and 5 μM gold (Fig. 1), expression of sMMO polypeptides decreased, and no sMMO activity was apparent (Fig. 2; see also Fig. S2 in the supplemental material). Further, the amount of copper associated with biomass increased significantly (Fig. 3). It appears that under these conditions, *M. trichosporium* OB3b was able to sequester copper and, by doing so, to prevent sMMO expression and activity. It is interesting, however, that a substantial amount of gold was still associated with the biomass. It appears that the endogenous methanobactin produced by *M. trichosporium* OB3b bound gold under these conditions, but the uptake of gold itself did not allow for sMMO expression. Rather, it appears that the absence of copper-metha-

nobactin complexes when only gold and copper were present enabled sMMO expression in the presence of copper. It is also interesting that the not only did *mmoX* expression increase in the presence of gold, but also expression of the gene encoding the precursor polypeptide of methanobactin, *mbnA*, increased, and such expression was not significantly different from that in cultures grown in the absence of copper and gold (Fig. 1C). It appears that *M. trichosporium* OB3b responded to the inability to collect copper in the presence of gold by increasing the production of methanobactin.

The addition of gold, although it clearly affected the expression and activity of sMMO as well expression of *mbnA*, had no discernible effect on the expression of *pmoA* in *M. trichosporium* OB3b. It is clear, then, that genes for both forms of MMO were expressed simultaneously in the presence of copper and gold, and it may be that both MMOs were active when *M. trichosporium* OB3b was grown in the presence of gold and copper. It is interesting that polypeptides of both sMMO and pMMO from the bacterial group of *Methylocystaceae* were found in the same sample of microbial communities as associated with the roots of field-grown rice (41). It may be that in many environments, both forms of MMO are simultaneously expressed, either by the same or by different members of the methanotrophic community. The data presented here suggest that such expression may be due in part to the presence of copper and other group A metals.

The finding that the presence of at least one group A metal, gold, can allow for expression and activity of sMMO in the presence of copper also suggests new strategies to manipulate methanotrophic activity in both natural and engineered environments. It may be desirable to have sMMO expressed due to its faster turnover rate, but inducing sMMO expression may be challenging if copper is present. It is difficult, if not practically impossible, to remove copper from a large complex environment. It may be much easier, however, to reduce copper uptake by adding a group A metal such that methanobactin binding of copper is limited. Certainly the use of gold for such a purpose is not feasible given its high cost. It may be that other group A metals, either singly or in combination, that provide an inexpensive means to selectively force methanotrophic communities to express sMMO can be identified. It is recommended that further field work be done integrating molecular and biochemical assays for detection of the expression and activity of both forms of MMO with more detailed geochemical characterization of metal speciation and concentration. Such work will help determine if there is any correlation between the presence of other metals that can compete with copper for binding to methanobactin and sMMO expression and activity.

Finally, it should be kept in mind that it is unclear how widespread this phenomenon may be; i.e., do all switchover methanotrophs express sMMO in the presence of metals that compete for binding to methanobactin, thereby limiting copper uptake? Do all forms of methanobactin exhibit the same ability to bind gold or other group A metals? Given the significant similarity between the known forms of methanobactin (23–26), it is plausible to presume that they would all bind gold to some degree, but the resultant impact on MMO expression is less clear. For example, some nonswitchover methanotrophs, i.e., those that can express only pMMO, are known to produce methanobactin (24, 25). If these strains are challenged with gold, it may be that their growth and activity may be inhibited, as they cannot express sMMO and are unable to take up sufficient copper to allow for optimal pMMO activity. Therefore, the presence of competing metals may serve to stimulate the survival of switchover methanotrophs *in situ*. It is suggested that future work consider the impact of competing metals for binding to methanobactin in mixed methanotrophic cultures.

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