Marker Exchange Mutagenesis of *mxoF*, Encoding the Large Subunit of the Mxo Methanol Dehydrogenase, in *Methylosinus trichosporium* OB3b

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Methanotrophs have remarkable redundancy in multiple steps of the central pathway of methane oxidation to carbon dioxide. For example, it has been known for over 30 years that two forms of methane monooxygenase, responsible for oxidizing methane to methanol, exist in methanotrophs, i.e., soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO), and that expression of these two forms is controlled by the availability of copper. Specifically, sMMO expression occurs in the absence of copper, while pMMO expression increases with increasing copper concentrations. More recently, it was discovered that multiple forms of methanol dehydrogenase (MeDH), Mxo MeDH and Xox MeDH, also exist in methanotrophs and that the expression of these alternative forms is regulated by the availability of cerium. That is, expression of Xox MeDH increases in the presence of cerium, while Mxo MeDH expression decreases in the presence of cerium. As it had been earlier concluded that pMMO and Mxo MeDH form a supercomplex in which electrons from Mxo MeDH are back donated to pMMO to drive the initial oxidation of methane, we speculated that Mxo MeDH could be rendered inactive through marker-exchange mutagenesis but growth on methane could still be possible if cerium was added to increase the expression of Xox MeDH under sMMO-expressing conditions. Here we report that *mxoF*, encoding the large subunit of Mxo MeDH, could indeed be knocked out in *Methylosinus trichosporium* OB3b, yet growth on methane was still possible, so long as cerium was added. Interestingly, growth of this mutant occurred in both the presence and the absence of copper, suggesting that Xox MeDH can replace Mxo MeDH regardless of the form of MMO expressed.

In methanotrophy, the central oxidation pathway of methane is well-known, with methane first being oxidized to methanol by methane monooxygenase (MMO) and methanol further being converted to formaldehyde by methanol dehydrogenase (MeDH). Subsequently, formaldehyde can be combined with either tetrahydrofolate or tetrahydromethanopterin to form methylene tetrahydrofolate and methylene tetrahydromethanopterin, respectively. Methylene tetrahydrofolate can then be assimilated into biomass via the serine cycle. Methylene tetrahydromethanopterin is oxidized to formate, which can be either converted to carbon dioxide via formate dehydrogenase or reduced to methylene tetrahydrofolate. It is speculated that such redundancy for the conversion of formaldehyde exists to control the buildup of this toxic intermediate (1–9).

What is less readily discerned from this simple pathway, however, is that substantial redundancy exists for many steps of the transformation of methane to carbon dioxide and not just the condensation of formaldehyde with either methylene tetrahydrofolate or methylene tetrahydromethanopterin. For example, the first step of methane oxidation (conversion of methane to methanol) can be carried out by two different forms of MMO that are regulated by the availability of copper, i.e., the copper switch. In the absence of copper, some methanotrophs synthesize an iron-containing cytoplasmic or soluble methane monooxygenase (sMMO). Most methanotrophs, however, can produce only a copper- and iron-containing membrane-bound or particulate methane monooxygenase (pMMO), and such expression increases with the increasing availability of copper (2, 10–12). It may be that such redundancy allows methanotrophs to survive under a

wide range of environmental conditions with significant variations in metal bioavailability.

More recently, methanotrophs have been shown to have multiple homologs of MeDH. The first and best-characterized MeDH is a heterotetrameric ($\alpha_2\beta_2$) pyrroloquinolone quinone (PQQ)-linked enzyme with two 66-kDa (α , or MxoF) subunits and two 8.5-kDa (β , or MxoI) subunits, i.e., Mxo MeDH. Mxo MeDH has been well characterized and is known to have calcium in its active site (13). Methanotrophs, however, have an alternative PQQ-linked MeDH with an alternative large subunit of 65 kDa, termed XoxF, and this has ~50% amino acid sequence identity to MxoF. This form of MeDH, Xox MeDH, can be composed of either XoxF only or XoxF associated with MxoI (14, 15). Unlike MxoF, multiple homologs of XoxF are often found in methanotrophs (1, 15). Xox MeDH, however, appears to not have calcium in its active site but has a rare earth element, such as cerium, lanthanum, or praseodymium, and from activity measurements, it appears that

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TABLE 1 Primers used in this study

Primer	Targeted gene	Sequence (5'–3') ^a	Reference or source
mxoF_OB3b_F3	<i>mxoF</i> (arm A)	GCTGCTGAATTCGGTATCCC	This study
mxoF_OB3b_R3		ATTTTGGATCCACGTCATAGGCGGTCATGTA	This study
mxoF_OB3b_F4		ATTTTGGATCCATCATTTGGCCAAGGACGTG	This study
mxoF_OB3b_R4		ATTTTAAAGCTTAGGTTACGTCGTCATAGGG	This study
qpmoA_FO	<i>pmoA</i>	TTCTGGGGCTGGACCTAYTTC	42
qpmoA_RO		CCGACAGCAGCAGGATGATG	
qmmoX_FO	<i>mmoX</i>	TCAACACCGATCTSAACAACG	42
qmmoX_RO		TCCAGATTCCRCCCCAATCC	
qxoxF1_FO	<i>xoxF1</i>	TCAAGGACAAGGTGTTTCGTC	20
qxoxF1_RO		CGAGCCGTCCTTGATGTTAT	
qxoxF2_FO	<i>xoxF2</i>	GCGCGAAGGATTGGGAATAT	20
qxoxF2_RO		GCCTCGTAATTCATGCACAG	

^a Underlined sequences represent different restriction sites. Y, S, and R, the IUPAC DNA codes for the C/T, C/G, and A/G nucleobases, respectively.

some forms of Xox MeDH may be catalytically superior to Mxa MeDH (16–19).

Recently, we have shown that for *Methylosinus trichosporium* OB3b, the expression of genes encoding polypeptides of Mxa MeDH and Xox MeDH vary with variations in the amounts of cerium. That is, expression of *mxoF* in *M. trichosporium* OB3b substantially decreased with increasing cerium concentrations, while expression of two alternate *xoxF* genes (*xoxF1* and *xoxF2*) significantly increased with increasing cerium concentrations. Changes in the levels of *mxoF* expression in response to cerium, however, occurred only in the absence of copper; i.e., when copper was also present, cerium had little effect on *mxoF* expression but still increased the levels of *xoxF1* and *xoxF2* expression (20). Based on these data, we concluded that (i) *M. trichosporium* OB3b does not need Mxa MeDH when utilizing sMMO for methane oxidation and (ii) these findings provide additional evidence supporting the conclusion that pMMO and Mxa MeDH form a supercomplex whereby electrons from methanol oxidation are back transferred to pMMO to drive the initial oxidation of methane to methanol (21–23).

If these conclusions are indeed correct, they would suggest that any attempt to knock out the expression of Mxa MeDH in *Methylosinus trichosporium* OB3b would be lethal either in the absence of cerium (as Xox MeDH expression and activity would be low) or in the presence of copper (as pMMO would be expressed and would require the formation of a supercomplex with Mxa MeDH for activity). Instead, such mutants could be created and maintained only in the presence of cerium and the absence of copper. Here we describe the construction and phenotype of a *M. trichosporium* OB3b mutant where *mxoF* has been knocked out using marker-exchange mutagenesis.

MATERIALS AND METHODS

Microbial growth conditions. Nitrate mineral salt (NMS) medium (24) was used to grow the *M. trichosporium* OB3b wild type and the mutant with an *mxoF* knockout (*mxoF::Gm^r*) with methane as the only growth substrate. Cultures were supplemented with copper (as CuCl₂) and/or cerium (as CeCl₃) from stock solutions prepared in >18 MΩ · cm H₂O. Copper and cerium stock solutions were filter sterilized using 0.2-μm-pore-size polyethersulfone membranes. *M. trichosporium* OB3b wild-type and *mxoF::Gm^r* cultures were grown at 30°C in 50 ml of NMS medium in 250 ml acid-washed Erlenmeyer flasks under constant shaking at 200 rpm. All chemicals used were of American Chemical Society grade or better.

For examination of the growth of the *mxoF::Gm^r* mutant in the pres-

ence of various copper and cerium concentrations, a starter culture grown in NMS medium with 25 μM cerium was used as an inoculum. During the late exponential phase, this culture was harvested by centrifugation at 5,000 × g for 15 min at 4°C. The cell pellet was washed twice in NMS medium (with no copper or cerium), and after each wash the cell pellet was resuspended in 50 ml NMS medium with no copper or cerium and centrifuged at 5,000 × g for 15 min at 4°C. After the second wash, the cell pellet was again resuspended in 50 ml NMS medium. The washed cell suspension was used as an inoculum (1:10 dilution) and inoculated into new cultures to create an initial optical density at 600 nm (OD₆₀₀) of ~0.05. Four different culture conditions were considered, and biological duplicates were used for each condition: (i) 0 μM copper (no copper was added) plus 0 μM cerium (no cerium was added), 0 μM copper plus 25 μM cerium, 10 μM copper plus 0 μM cerium, and 10 μM copper plus 25 μM cerium. After these cultures reached the stationary phase, they were used as the inocula (1:10 dilution) to start a new set of identical cultures. After the second set of cultures reached the stationary phase, a third set of cultures was started, again using the previous cultures as the inocula (1:10 dilution). The cells from the third set of serial transfers were harvested for the analyses described below.

Escherichia coli was grown in LB medium. Kanamycin (final concentration, 25 μg · ml⁻¹) or gentamicin (final concentration, 5 μg · ml⁻¹) was used for maintaining *E. coli* cells containing mutant construct plasmids, while only gentamicin (final concentration, 2.5 μg · ml⁻¹) was used for maintaining all *mxoF::Gm^r* mutant cultures after the successful knockout of *mxoF*.

Construction of the *mxoF::Gm^r* mutant of *M. trichosporium* OB3b. For the construction of the *mxoF::Gm^r* mutant, a double homologous recombination method was used, as described earlier (25). Briefly two DNA fragments from the 5' and 3' ends (arm A and arm B, respectively) of the *mxoF* gene of *M. trichosporium* OB3b were amplified by PCR. Specific primers (Table 1) were designed to amplify arms A and B and to introduce EcoRI, BamHI, and HindIII restriction site sequences, to facilitate directional cloning into the pK18mobsacB suicide vector. The PCR products of arms A and B were digested with BamHI and gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions. A single DNA fragment, fragment AB, was constructed after the ligation of the two arms, and the ligation product was used as the template for PCR amplification using the forward primer specific for arm A and the reverse primer specific for arm B. After digestion with EcoRI and HindIII and gel purification using the QIAquick gel extraction kit (Qiagen, Valencia, CA), fragment AB was cloned into pK18mobsacB, yielding construct pmxoF_AB. A gentamicin resistance cassette (865 bp) was obtained after digestion of plasmid p34S-Gm with BamHI, and this cassette was cloned via the BamHI site between products of arms A and B in plasmid pmxoF_AB. The resulting plasmid was the final construct and was named pMFU01. Plasmid pMFU01 was trans-

ferred to chemically competent cells of *E. coli* S17.1 λ pir (26) by the heat shock method. Finally, the pMFU01 construct was transferred to *M. trichosporium* OB3b via conjugation between the donor (*E. coli* S17.1 λ pir containing the pMFU01 construct) and the recipient (*M. trichosporium* OB3b) strains according to previously described methods (27). The transconjugants were selected on NMS agar plates containing $5 \mu\text{g} \cdot \text{ml}^{-1}$ gentamicin and $25 \mu\text{M}$ cerium. To ensure the absence of *E. coli* in the background, colonies of the resultant *M. trichosporium* OB3b *mxoF::Gm^r* mutant were plated and then cultured in flasks containing NMS medium supplemented with $10 \mu\text{g} \cdot \text{ml}^{-1}$ nalidixic acid, $5 \mu\text{g} \cdot \text{ml}^{-1}$ gentamicin, and $25 \mu\text{M}$ cerium. PCR and sequencing analyses were used to confirm recombination in the double homologous recombinants and the genotype of the crossover mutants.

Naphthalene assay. The activity of sMMO in the *mxoF::Gm^r* mutant was measured by the naphthalene assay developed by Brusseau et al. (28) with some modifications. Briefly, a few flakes of naphthalene were added to 1.6 ml of cells in a 2-ml screw-cap tube during harvesting of the cultures. The cells were then incubated for 1 h at 30°C with constant shaking at 200 rpm. After centrifugation at $6,000 \times g$ for 5 min, $130 \mu\text{l}$ of 4.21 mM Fast Blue B (tetrazotized *o*-dianisidine) was added to 1.3 ml of the supernatant.

Protein quantification. Total cell protein was quantified using the Bradford assay (Bio-Rad, Hercules, CA) as described earlier (25). Briefly, 5 ml of each culture was concentrated to 1 ml and digested in 2 M NaOH at 98°C for 15 min. Protein concentrations were determined using the Bradford assay according to the manufacturer's instructions. After plotting of the protein concentrations versus the OD_{600} s of the different cultures, a linear regression was obtained and showed that an OD_{600} of 1.0 is equal to $850 \mu\text{g}$ of protein per ml ($R^2 = 0.995$). The protein concentrations from all cultures were calculated using this correlation.

Metal measurements. After growth, the concentrations of cerium and copper associated with the biomass and those remaining in the supernatant were measured as described previously (20). Briefly, 20-ml cultures of the *mxoF::Gm^r* mutant were harvested by centrifugation at $5,000 \times g$ for 10 min at 4°C . The cell pellets were washed in 20 ml fresh NMS medium and centrifuged again at $5,000 \times g$ for 10 min at 4°C . The cell pellets were then resuspended in 1 ml of fresh NMS medium and stored at -80°C . For performing metal analyses, all samples were thawed at room temperature. One milliliter of 70% (vol/vol) HNO_3 was added to each cell suspension, and then the cell suspension was digested for 2 h at 95°C . Digested cell suspensions were diluted in NMS medium so that the final HNO_3 concentration was 2%. Subsequent metal analyses were performed using inductively coupled plasma mass spectrometry (ICP-MS 7900; Agilent Technologies, Santa Clara, CA).

Extraction of DNA and RNA. Late-exponential-phase cultures of the *mxoF::Gm^r* mutant grown under different conditions were harvested for both DNA and RNA extraction. Total DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA extraction was performed as described earlier (25). Briefly, cultures (9 ml) were harvested by the addition of 1 ml of stop solution (5% buffer-equilibrated phenol [pH 7.3] in ethanol) and centrifugation at $5,000 \times g$ for 10 min at 4°C . The supernatants were discarded, while the cell pellets were resuspended in 0.75 ml of extraction buffer. After addition of 0.5 g of 0.1-mm zirconia-silica beads (Biospec Products, Bartlesville, OK), $35 \mu\text{l}$ of 20% sodium dodecyl sulfate (SDS), and $35 \mu\text{l}$ of 20% Sarkosyl, the samples were subjected to bead beating (1 min at 4,800 rpm) in 2-ml plastic tubes. RNA extractions were then performed as described previously (25). Total extracted RNA was subjected to RNase-free DNase treatment until it was free of DNA contamination, as determined via PCR amplification of the 16S rRNA gene. The purified RNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). RNA samples were stored at -80°C and used for cDNA synthesis within 2 days of extraction. Reverse transcription (RT) was performed on DNA-free total RNA (500 ng) to synthesize cDNA

FIG 1 Verification of knockout of *mxoF* in *M. trichosporium* OB3b by PCR. (A) PCR of *mxoF*. Lane 1, PCR of *mxoF* from *M. trichosporium* OB3b *mxoF::Gm^r*; lane 2, PCR of *mxoF* from *M. trichosporium* OB3b wild type; lane 3, PCR of *mxoF* from plasmid construct pMFU01 (positive control); lane 4, PCR of *mxoF* from nuclease-free water (negative control); lane M, molecular size markers. (B) PCR of the pk18mobsacB backbone. Lane 1, PCR of the pk18mobsacB backbone in *M. trichosporium* OB3b *mxoF::Gm^r*; lane 2, PCR of the pk18mobsacB backbone in the *M. trichosporium* OB3b wild type; lane 3, PCR of the pk18mobsacB backbone in pMFU01 (positive control); lane 4, PCR of the pk18mobsacB backbone in nuclease-free water (negative control); lane M, molecular size markers.

using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

qPCR analyses. Gene-specific primers (Table 1) were used for quantitative PCR (qPCR) and reverse transcription-quantitative PCR (RT-qPCR) analyses of *pmoA*, *mmoX*, *xoxF1*, and *xoxF2* in cultures of the *mxoF::Gm^r* mutant grown under different concentrations of copper and cerium. The specificities of these primers were verified by gel electrophoresis and sequencing analyses. qPCRs were performed in 96-well reaction PCR plates using a CFX Connect real-time PCR detection system (Bio-Rad). Each qPCR mixture (20 μl) contained 0.8 μl cDNA/DNA, $1 \times$ iTaq Universal SYBR green supermix (Bio-Rad, Hercules, CA), 0.5 μM each forward and reverse primers, and nuclease-free sterile water (Ambion/Life Technologies, Grand Island, NY). A three-step program for qPCR consisted of 40 cycles of denaturation (95°C for 20 s), annealing (58°C for 20 s), and extension (68°C for 30 s) after an initial denaturation at 95°C for 3 min. After the completion of the amplification cycles, the qPCR products were subjected to melting curve analysis at temperatures ranging from 55°C to 95°C to confirm their specificity. The amplification threshold cycle (C_T) values were then imported from CFX Manager software (Bio-Rad, Hercules, CA) into Microsoft Excel software to quantify the expression of the different genes. The gene expression levels were calculated in terms of the transcript number per copy number determined from the RNA and DNA extracted from the same culture at the same growth point. Gene transcript and copy numbers were calculated from calibration curves developed with plasmid preparations with known copy numbers for each gene examined.

RESULTS

Using marker-exchange protocols, *mxoF* was successfully knocked out by growing transconjugants on NMS medium with no added copper and $25 \mu\text{M}$ cerium. Under these conditions, a single colony with a double homologous recombination event whereby a gentamicin cassette was inserted in *mxoF* was identified, as shown in Fig. 1. This was confirmed via sequencing, as well as verification that the mutant was resistant to gentamicin and sucrose but sensitive to kanamycin (data not shown).

After isolation of the *mxoF::Gm^r* mutant via the use of selective growth conditions on plates, the mutant was then cultured in liquid culture using NMS medium with $0 \mu\text{M}$ copper (no copper was added) and $25 \mu\text{M}$ cerium. After an OD_{600} of ~ 0.5 was

FIG 2 Growth of *M. trichosporium* OB3b *mxoF::Gm^r* with various copper and cerium concentrations. ○, 0 μ M copper plus 0 μ M cerium; ●, 0 μ M copper plus 25 μ M cerium; □, 10 μ M copper plus 0 μ M cerium; ■, 10 μ M copper plus 25 μ M cerium. Errors bars represent the ranges for duplicate samples.

reached, the initial 50 ml of culture was washed, resuspended in 50 ml of fresh NMS medium with no cerium or copper, and then transferred at a 1:10 ratio to NMS medium containing either 0 μ M copper plus 0 μ M cerium, 0 μ M copper plus 25 μ M cerium, 10 μ M copper plus 0 μ M cerium, or 10 μ M copper plus 25 μ M cerium. As can be seen in Fig. 2, the *mxoF::Gm^r* mutant grew, to our surprise, under all conditions, but with the longest lag phases being for the mutant grown in the presence of 10 μ M copper with or without 25 μ M cerium and the lowest overall growth being in the presence of 10 μ M copper and the absence of cerium.

Given the subsequent initial growth under all conditions, particularly in the absence of cerium, it was speculated that the *mxoF::Gm^r* mutant was able to grow due to the transfer of not only cells but also substantial amounts of cerium from the initial seed culture, as it was found earlier that >98% of the added cerium is associated with biomass (20). To test this hypothesis, these cultures were transferred at a 1:10 ratio to fresh identical medium to limit the transfer of cerium and growth was monitored (Fig. 2). As can be seen after this transfer at ~150 h (transfer 1), the mutant grew best in the absence of copper and the presence of 25 μ M cerium. Growth was also evident in the presence 10 μ M copper and 25 μ M cerium, but the lag phase was extended compared to the lag phase obtained when no copper was added. Some growth

was seen for the mutant grown in the absence of both copper and cerium, but no growth was evident in the presence of 10 μ M copper and no added cerium.

Those cultures that grew after transfer 1 were transferred again at a 1:10 ratio at ~275 h (transfer 2). As shown in Fig. 2, after transfer 2, only the *mxoF::Gm^r* mutant cultivated in the presence of cerium showed any regrowth, but the presence or absence of copper had little effect, although the *mxoF::Gm^r* mutant grown in the presence of 10 μ M copper again had a longer lag phase than the mutant grown in the absence of copper.

These cultures were then harvested and examined for metal uptake as well as the expression of select genes and sMMO activity. As can be seen in Fig. 3A, there was no significant difference in the amount of cerium associated with the mutant when it was grown in the presence of either 0 μ M copper plus 25 μ M cerium or 10 μ M copper plus 25 μ M cerium, suggesting that copper had no effect on the ability of the *mxoF::Gm^r* mutant to sequester cerium. There was a substantial increase in the amount of copper associated with mutant biomass, however, when 10 μ M copper was added in addition to 25 μ M cerium ($P = 1 \times 10^{-2}$; Fig. 3B), also suggesting that the presence of cerium had no effect on the ability of the *mxoF::Gm^r* mutant to sequester copper.

Interestingly, when the *mxoF::Gm^r* mutant was grown and

FIG 3 Cerium (A) and copper (B) associated with the biomass of *M. trichosporium* OB3b *mxoF::Gm^r* grown in the presence of 25 μ M cerium and either 0 or 10 μ M copper. Errors bars represent the ranges for duplicate samples. The values for columns within each plot labeled by different letters are significantly different ($P < 0.05$).

FIG 4 RT-qPCR of *pmoA* (A), *mmoX* (B), *xoxF1* (C), and *xoxF2* (D) in the *M. trichosporium* OB3b *mxoF*::Gm^r mutant grown in the presence of 25 μM cerium and either 0 or 10 μM copper. Errors bars represent the ranges for duplicate samples. The values for columns within each plot labeled by different letters are significantly different ($P < 0.05$).

multiply transferred in the presence of 0 μM copper plus 25 μM cerium or 10 μM copper plus 25 μM cerium, *pmoA* and *mmoX* expression was dependent on the availability of copper, indicating that the copper switch was still operational, even though *mxoF* was knocked out (Fig. 4A and B). That is, the level of *pmoA* expression increased over 30-fold when copper was added ($P = 3 \times 10^{-3}$), while the level of *mmoX* expression decreased over 2,000-fold ($P = 0.02$). Further, the *mxoF*::Gm^r mutant relied on pMMO activity to grow in the presence of copper, as evidenced by the lack of any measureable sMMO activity using the naphthalene assay (Fig. 5). Finally, the expression of *xoxF1* and *xoxF2* did not change significantly as the amount of copper was varied in the presence of cerium (Fig. 4C and D).

DISCUSSION

We report here the successful knockout of *mxoF*, encoding the large subunit of the Mxa methanol dehydrogenase, in any methanotroph. Previously, it was thought to be impossible to knock out any component of the Mxa MeDH in a methanotroph and grow the resulting mutant on methane. The success described here results from the finding that at least some methanotrophs have an alternative methanol dehydrogenase, Xox MeDH, and that the expression and activity of Xox MeDH are dependent on the availability of cerium (16–20). By adding cerium to the NMS medium used to screen for transconjugants, we were able to successfully knock out *mxoF* yet still have growth of the mutant on methane.

Based on our earlier findings of cerium and copper regulation

of the expression of Mxa MeDH and Xox MeDH (20), coupled with the conclusion of others that pMMO forms a supercomplex with Mxa MeDH (21, 22), we speculated that the *mxoF*::Gm^r mutant would be able to grow under sMMO-expressing conditions,

FIG 5 sMMO oxidation of naphthalene in *M. trichosporium* OB3b *mxoF*::Gm^r grown in the presence of either 0 μM copper plus 25 μM cerium (A) or 10 μM copper plus 25 μM cerium (B).

so long as cerium was present, but not pMMO-expressing conditions, regardless of the presence or absence of cerium, as no functional pMMO-Mxa MeDH supercomplex would be formed. As expected, the *mxoF::Gm^r* mutant did not grow in the absence of cerium and copper after two transfers (Fig. 2; such transfers served to dilute the transfer of cerium from the initial inoculum). The mutant, however, did grow well after multiple transfers in the presence of copper, so long as cerium was also added. From these findings and the fact that (i) the *mxoF::Gm^r* mutant sequestered copper and (ii) the copper switch was evident in the mutant (Fig. 3 to 5), it appears that Xox MeDH can replace Mxa MeDH, regardless of the form of MMO expressed, and that the MeDH-pMMO supercomplex is not necessary for growth. It is interesting to note that although the mutant did grow in the presence of copper and cerium, these cultures initially exhibited a longer lag than the culture grown solely in the presence of cerium (Fig. 2), suggesting that the mutant had to adapt its metabolism to a more significant degree in the presence of copper than in its absence to grow on methane. At this time, it is unclear what the cause of this lag might be. Two possibilities are that (i) Xox MeDH may associate more weakly with pMMO, forming a less stable supercomplex, and/or (ii) the electron transport pathway requires some manipulation to effectively redirect the reducing equivalents generated from carbon oxidation to pMMO. In any event, a recent metabolic modeling study indicates that direct coupling between methane and methanol oxidation is important for pMMO activity (23), and it may be that the level of such coupling between pMMO and Xox MeDH is reduced compared to that between pMMO and Mxa MeDH.

From the data presented here, though, it is clear that methanotrophs have remarkable redundancy in the central pathway of methane oxidation. With the mutant, we can now examine other facets of methanotrophy; e.g., it has been reported that at least some Xox MeDHs are catalytically superior to Mxa MeDH (17, 19). This is possible, as lanthanide(III) ions, such as Ce(III), are much stronger Lewis acids than Ca(II) and thus are likely to be much more efficient catalysts for hydrolysis (29). With the mutant, we can now more easily isolate and purify Xox MeDH for kinetic and structural analyses while avoiding possible artifacts resulting from the presence of Mxa MeDH.

Finally, these data indicate that methanotrophs have some mechanism(s) to collect cerium. This mechanism is unclear, but there are several possibilities, including (i) adventitious leaching of cations, such as cerium, through the secretion of low-molecular-weight organic acids, inorganic acids, and/or metal-binding compounds, such as chalkophores or siderophores (30–34); (ii) secretion of a specific cerium-binding compound akin to chalkophores for copper and siderophores for iron; and (iii) uptake of cerium as cerium phosphate via systems such as the P_i transport system (Pit). This low-affinity, high-velocity system is used by many microbes for phosphate uptake and has been shown to control the accumulation of metals, such as zinc, calcium, and magnesium (35–39).

None of these possibilities can be excluded at this time. That is, methanotrophs are known to produce both chalkophores and siderophores (40), and these may be partially responsible for increasing the bioavailability of cerium. Further, it was discovered over 40 years ago that methanotrophs produce a number of water-soluble pigments that may include novel metal-binding compounds (24). Methanotrophs have also been shown to produce low-molecular-

weight organic acids under microaerobic conditions (41), suggesting that this may play a role in the leaching of cerium. Finally, a preliminary review of the genome of *M. trichosporium* OB3b indicates that this strain does indeed have the P_i transport system (data not shown). It may be that one or some combination of these systems is used for cerium collection by methanotrophs.

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