

Lanthanide-Dependent Regulation of Methanol Oxidation Systems in *Methylobacterium extorquens* AM1 and Their Contribution to Methanol Growth

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ABSTRACT

Methylobacterium extorquens AM1 has two distinct types of methanol dehydrogenase (MeDH) enzymes that catalyze the oxidation of methanol to formaldehyde. MxaFI-MeDH requires pyrroloquinoline quinone (PQQ) and Ca in its active site, while XoxF-MeDH requires PQQ and lanthanides, such as Ce and La. Using MeDH mutant strains to conduct growth analysis and MeDH activity assays, we demonstrate that *M. extorquens* AM1 has at least one additional lanthanide-dependent methanol oxidation system contributing to methanol growth. Additionally, the abilities of different lanthanides to support growth were tested and strongly suggest that both XoxF and the unknown methanol oxidation system are able to use La, Ce, Pr, Nd, and, to some extent, Sm. Further, growth analysis using increasing La concentrations showed that maximum growth rate and yield were achieved at and above 1 μ M La, while concentrations as low as 2.5 nM allowed growth at a reduced rate. Contrary to published data, we show that addition of exogenous lanthanides results in differential expression from the *xoxI* and *mxo* promoters, upregulating genes in the *xoxI* operon and repressing genes in the *mxo* operon. Using transcriptional reporter fusions, intermediate expression from both the *mxo* and *xoxI* promoters was detected when 50 to 100 nM La was added to the growth medium, suggesting that a condition may exist under which *M. extorquens* AM1 is able to utilize both enzymes simultaneously. Together, these results suggest that *M. extorquens* AM1 actively senses and responds to lanthanide availability, preferentially utilizing the lanthanide-dependent MeDHs when possible.

IMPORTANCE

The biological role of lanthanides is a nascent field of study with tremendous potential to impact many areas in biology. Our studies demonstrate that there is at least one additional lanthanide-dependent methanol oxidation system, distinct from the MxaFI and XoxF MeDHs, that may aid in classifying additional environmental organisms as methylotrophs. Further, our data suggest that *M. extorquens* AM1 has a mechanism to regulate which MeDH is transcribed, depending on the presence or absence of lanthanides. While the mechanism controlling differential regulation is not yet understood, further research into how methylotrophs obtain and use lanthanides will facilitate their cultivation in the laboratory and their use as a biomining and biorecycling strategy for recovery of these commercially valuable rare-earth elements.

Methylotrophs have gained worldwide interest as platforms for the production of value-added chemicals from single-carbon compounds, turning atmospheric pollutants like methane and methanol into green chemicals, including biofuels and biodegradable plastics (1–5). A key step in this process is the oxidation of methanol to formaldehyde, which is carried out by different enzymes, including methanol dehydrogenase (MeDH) and alcohol oxidase, depending on the specific methylotroph (6, 7). Recently, it was discovered that some types of MeDHs require rare-earth elements, specifically lanthanides, as cofactors (8–11).

Rare-earth elements include 15 lanthanides (lanthanum [La], cerium [Ce], praseodymium [Pr], neodymium [Nd], promethium [Pm], samarium [Sm], europium [Eu], gadolinium [Gd], terbium [Tb], dysprosium [Dy], holmium [Ho], erbium [Er], thulium [Tm], ytterbium [Yb], and lutetium [Lu]) and two chemically similar elements (scandium [Sc] and yttrium [Y]). The term “rare earth” is deceptive, as these lanthanide elements are relatively abundant in the Earth’s crust, found at levels similar to those seen for copper and zinc (Ce, 66 ppm; La 39 ppm; Cu, 60 ppm, Zn, 70 ppm) (12). However, lanthanides are highly insoluble and are rarely found in pure form (13). Because of their spectroscopic,

superconductive, and magnetic properties, lanthanides are used in lasers, wind turbines, hybrid car batteries, and military weaponry, along with many of our “everyday use” items, such as smart phones and computers (14, 15). While commercially important, the low bioavailability of lanthanides prompted the belief that biology had evolved to effectively ignore these elements (16). However, in some environments, such as volcanic mud pots, concentrations of soluble lanthanides can reach the low micromolar range (8). Studies have shown that plants can effectively concentrate lanthanides like La from the soil, with measurements ranging

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from 0.18 to 3.1 $\mu\text{g La/g}$ dry leaf mass (11). These studies have had profound impacts on the study of methylotrophic organisms, as different methylotrophic bacteria have now been shown to use lanthanides directly for MeDH activity and addition of lanthanides to growth media has facilitated the growth of organisms that had been difficult or impossible to culture before (8, 11, 17).

Until recently, it was believed that in *Methylobacterium extorquens* AM1, a model organism for understanding methylotrophic growth, methanol oxidation was catalyzed solely by the extensively studied Ca- and pyrroloquinoline quinone (PQQ)-dependent MeDH encoded by the *mxoA* genes (18). Each large subunit (MxoA) contains Ca and PQQ, both of which are essential for the oxidation of methanol to formaldehyde in the periplasmic space. The *xoxF* gene was first identified in *M. extorquens* AM1 over a decade ago as a predicted PQQ-dependent periplasmic alcohol dehydrogenase, though its role in methylotrophy was unclear at the time (19, 20). When completed, the genome sequence of *M. extorquens* AM1 revealed a second *xoxF* gene, *xoxF2*, which, like *xoxF1*, encodes a protein that shares 50% amino acid identity with the MxoA subunit of the Ca-dependent MeDH (21, 22).

After the initial discovery of *xoxF1*, the physiological role of the XoxF proteins remained unknown for ~ 15 years. In 2011, XoxF1 was purified and demonstrated a low rate of methanol oxidation *in vitro* ($V_{\text{max}} = 0.015 \text{ U/mg}$) (23), yet overexpression of *xoxF* was unable to functionally complement an *mxoA* mutant *in vivo* (22), complicating the interpretation of these findings. Intriguingly, the *xoxF* genes were also shown to be required for expression of the *mxoA* genes, suggesting that XoxF has a regulatory role, in addition to a catalytic role, in *M. extorquens* AM1 (22). A breakthrough as to the enzymatic role of XoxF came when Hibi et al. grew *Methylobacterium radiodurans* in medium containing La and purified MeDH by following its activity (24). N-terminal sequencing revealed that XoxF and not MxoA was responsible for this MeDH activity (24). This suggested that XoxF might function as a La-dependent MeDH, but there was still a lack of direct proof. It was formally possible that XoxF-MeDH required Ca for the oxidation of methanol but was upregulated in the presence of La. In 2012, this was resolved when Nakagawa et al. demonstrated that MeDH, purified from *M. extorquens* AM1 cells grown in medium containing La, consisted of a XoxF dimer containing 1.24 atoms of La and lacked Ca (11). Since these findings, XoxF has been purified from a variety of methylotrophs, displaying differences in subunit composition, optimal enzyme assay parameters, and oxidation products depending on the organism studied (9, 10, 25). In 2014, Pol et al. addressed the biochemical promiscuity of XoxF MeDHs by determining that a variety of lanthanides, including La, Ce, Pr, and Nd, could support methanol-dependent growth of *Methylacidiphilum fumariolicum* SolV (8).

Our current understanding of *mxoA* and *xox* gene regulation is incomplete. In *M. extorquens* AM1, two two-component systems (MxcQE and MxbDM) and an additional response regulator (MxB) are required for expression of the *mxoA* operon, though it is not known if the requirement for these regulators is direct or indirect or what is being sensed by the systems (22, 26, 27). Additionally, the MxbDM two-component system is required for repression of the *xox1* operon in the absence of lanthanides, while nothing is known about the regulation of *xoxF2* expression (22). Among methylotrophic bacteria that contain both *mxoA* and *xoxF* homologs, the presence and copy numbers of these regulator genes vary, making it difficult to define a general requirement and

role for the regulators in methylotrophy (25). The studies addressing how methylotrophic bacteria sense lanthanides and differentially regulate expression of the *mxoA* and *xox* genes are limited and conflicting. In *M. extorquens* AM1, it was initially reported that La does not affect expression of the *mxoA* or *xox1* operon genes (11), which conflicted with the subsequent findings described by Skovran and Martinez-Gomez (17). Following these reports, Farhan et al. showed that in the methanotrophic bacterium *Methylosinus trichosporium* OB3b, exogenous Ce upregulates expression of both *xoxF* copies in its genome and represses expression of the *mxoA* genes (28). Interestingly, significant repression of *mxoA* and *mxoB* was seen only in the absence of copper, which controls differential expression of the soluble and particulate methane monooxygenases that oxidize methane to methanol (29). *M. trichosporium* OB3b does not contain obvious homologs of *mxoD* and *mxoM* but does contain a sensor kinase (*mxoY*) and a response regulator gene (*mxoB*) upstream of the *mxoA* genes whose products share 34% and 36% identity with MxcQ and MxB in *M. extorquens* AM1, respectively. Whether these regulators are required for differential expression of the *xox* and *mxoA* genes is not yet known.

In this study, we describe the range of lanthanides that allow lanthanide-dependent methanol growth in *M. extorquens* AM1 and describe the contribution by each known MeDH to growth using MeDH mutant strains. Further, we report for the first time the existence of an additional, unidentified lanthanide-dependent methanol oxidation system. We also refute the conclusions of Nakagawa et al. regarding expression of the *mxoA* and *xox1* operon genes (11), as we show that differential expression from the *mxoA* and *xox1* promoters is very sensitive to the presence of La, Ce, Pr, and Nd. While it is clear that lanthanides differentially control expression of the *xoxF1* and *mxoA* genes, the regulatory network mediating this differential expression is complex and poorly understood and has been investigated only in the absence of lanthanides (22). Once it is understood how methylotrophs are able to sense and acquire lanthanides, they may be better engineered as alternative biomining and biorecycling strategies for recovery of these highly valued elements from lanthanide ores and discarded items, such as computer magnets and hybrid car batteries.

MATERIALS AND METHODS

Chemicals. Chemicals were purchased through Fisher Scientific (Pittsburgh, PA) unless otherwise stated. All lanthanides were used in their chloride salt forms for the preparation of stock solutions and were in the 3+ oxidation state.

Strain construction. The strains and plasmids used in this study are listed in Table 1. Plasmids were conjugated into *M. extorquens* AM1 via biparental matings using the *Escherichia coli* S17-1 helper strain (30) or via triparental matings using *E. coli* TOP10 (Invitrogen, Carlsbad, CA) and the *E. coli* helper strain containing the conjugative plasmid pRK2013 (31), as previously described (32), except that conjugation took place directly on the surface of nutrient agar instead of on a 45- μm membrane filter.

All *M. extorquens* AM1 strains used in this study (including those previously published) were created from identical wild-type frozen stocks. Null mutations in *xoxF1* and *xoxF2* were constructed in the wild type and *mxoA* deletion strains using the allelic marker exchange suicide vectors pAYC61 (20, 33) and pCM184 (34), respectively. The kanamycin (Km) resistance cassette in the *mxoA xoxF2::Km* mutant strain was deleted using the *cre* expression plasmid pCM157, as previously described (34). The Km insertion in *xoxF1* and the Km deletion in *xoxF2* were verified by diagnostic colony PCR. The expected presence or absence of *mxoA*, *xoxF1*, and *xoxF2* was verified in all the strains. Complementation of the *xoxF* and

TABLE 1 Strains and plasmids used in the study

Strain or plasmid	Description	Source or reference
<i>M. extorquens</i> ^a		
AM1	Rif ^r derivative (wild type)	43
CM194.1	$\Delta mxaF$	44
ES890	<i>sox1</i> ::Km	22
ES970	$\Delta sox2$	22
ES1022	$\Delta sox2 sox1$::Km	22
ES1017	$\Delta mxaF sox1$::Km	This study
ES2288	$\Delta mxaF \Delta sox2$	This study
ES1100	$\Delta mxaF \Delta sox2 sox1$::Km	22
<i>E. coli</i>		
TOP10	Competent cells for cloning (Sm ^r)	Invitrogen
S17-1	Helper strain for conjugation (Tp ^r Sm ^r)	30
Plasmids		
pAYC61	Allelic exchange suicide vector (Tc ^r Ap ^r)	33
pAP5	Promoterless <i>venus</i> fusion vector constructed from pCM62 (Tc ^r)	22
pCM157	<i>cre</i> expression vector (Tc ^r)	34
pCM184	Allelic exchange suicide vector (Km ^r Tc ^r Ap ^r)	34
pES134-135	pCM184 with <i>sox2</i> upstream and downstream flanks (Km ^r Tc ^r Ap ^r)	22
pES502	pAP5 containing the <i>mxs</i> promoter region (Tc ^r)	22
pES503	pAP5 containing the <i>sox1</i> promoter region (Tc ^r)	22
pHV-F2	pAP5 containing the <i>sox2</i> promoter region (Tc ^r)	This study
pLC6168	pAYC61 with <i>sox1</i> upstream and downstream flanks (Km ^r Tc ^r Ap ^r)	20
pRK2013	Conjugative helper plasmid (Km ^r)	31

^a All *M. extorquens* AM1 strains used in the study are isogenic.

mxsF single mutants and the *sox1 sox2* double-mutant strains was previously described (22, 35).

Transcriptional reporter fusion vectors were constructed using the multicopy plasmid pAP5 containing a modified version of yellow fluorescent protein, Venus, as previously described (22). The promoter regions of the *mxs* and *sox1* operons were amplified and cloned into the AclI restriction site using the following primer pairs to create pES502 and pES503, respectively: pAF-4538BEGAcII, TGAACGTTAGT TGTGCGCGTTCAGCATCACG, and pAF-4538EndAcII, TGAACGTT CGAGGAGCCGATGATGACCTTGT for *mxs* and pF1-1740BEGAcII, TGAACGTTCCGGCATCGTCCGCTCAGTAGAATC, and pF1-1740EndAcII, TGAACGTTTTCTGCCGGACTTCAGGTCGTAG, for *sox1*. Correct orientation of the promoter was confirmed by diagnostic PCR and sequencing. The primers pF2_2757BEGAcII, TGAACGTTAGTGGCGGTGCAG GACCACGAA, and pF2_2757EndEcoRI, TCGAATTCGACCAGTTGAC CTTGCCGGTCTT, were used to amplify the promoter region of *sox2*, followed by digestion with AclI and EcoRI and ligation into pAP5 to create pHV-F2.

Growth conditions. *M. extorquens* AM1 strains were grown at 29°C in *Methylobacterium* PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (MP) medium (36), except that FeSO₄ was omitted from the C7 metal mixture (citrate-chelated trace element solution containing 7 metals) and prepared separately. Succinate (0.4%) and/or methanol (0.5%) was added as a carbon source(s) to media containing calcium chloride (20 μM) and/or lanthanide chlorides (2.5 nM to 20 μM). Difco nutrient agar was used for bi- and triparental matings. *E. coli* strains were cultured at 37°C on Luria-Bertani (LB) agar. When appropriate, media were supplemented with antibiotics at the following concentrations: tetracycline (Tc) at 5 μg/ml for MP medium and 10 μg/ml for LB medium, Km at 50 μg/ml, and rifamycin (Rif) at 50 μg/ml.

Preparation of glassware. Even after washing with dilute acid, trace lanthanides can contaminate glassware at concentrations that vary by batch (8). In our studies, this resulted in variation in the levels of expression from the *mxs* and *sox1* promoters and variation in the growth of the MeDH mutant strains. Trace lanthanides were removed from the glassware by pregrow-

ing wild-type *M. extorquens* AM1 to full density in MP methanol medium lacking lanthanides. The glassware was then washed with deionized water and autoclaved for use in subsequent experiments. When the glassware was prepared in this manner, experimental results were reproducible and variation between replicates was minimal.

Growth curve analysis. Cells were grown at 29°C in borosilicate glass tubes (2-ml cultures in 16- by 125-mm tubes or 6-ml cultures in 20- by 150-mm tubes) placed in custom-built culture tube rack holders angled at 60° to achieve maximum aeration. The cultures were shaken at 180 rpm in an Excella E25 shaking incubator (New Brunswick Scientific, Edison, NJ). *M. extorquens* AM1 strains were grown to late exponential phase in 2 ml of MP succinate medium without exogenous Ca or lanthanides and then subcultured (200 μl) into 6 ml MP methanol medium containing or lacking exogenous Ca or lanthanides as indicated in the text. Optical density measurements were recorded at 600 nm (OD₆₀₀) using a Spectronic 20D spectrophotometer (Milton Roy Company, Warminster, PA) adapted to read absorbance in 20- by 150-mm tubes.

Transcriptional reporter fusion assays. Cells were grown in culture tubes as described above with addition of Tc to the MP medium. When the cells reached mid-exponential phase (OD₆₀₀, ~0.6), a 200-μl aliquot was removed, and the relative fluorescence units (RFU) were measured in optical-bottom black 96-well plates using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). The excitation and emission spectra used were 490 nm and 540 nm, respectively. The OD₆₀₀ measurements from the Spectramax M2 plate reader were used to normalize fluorescence to culture density (RFU/OD₆₀₀).

Methanol dehydrogenase assays. To prepare cell extracts, 2 ml of biological duplicates grown to mid-exponential phase was used to inoculate 50 ml of MP methanol medium (36) containing or lacking 2 μM La. Cells were harvested at an OD₆₀₀ of 0.8 to 1.2 by centrifugation at 4,729 × g at 4°C for 10 min. The cells were then washed with 100 mM Tris-HCl buffer at pH 9 and lysed with a French press. Cell debris was removed by centrifugation for 2 min at 28,078 × g and 4°C. The protein concentration was determined by the bicinchoninic acid (BCA) method (Sigma-Aldrich, St. Louis, MO).

MeDH activity was measured in cell extracts according to the

method of Anthony and Zatman (37) with minor modifications: the reaction mixture (final volume, 200 μ l) was prepared in 100 mM Tris-HCl at pH 9 and contained 15 mM NH_4Cl , 200 μM KCN, 500 μM phenazine methosulfate (PMS), 300 μM 2,6-dichlorophenol-indol-phenol (DCPIP), and cell extract (2.4 to 8.4 $\mu\text{g}/\mu\text{l}$ protein). Auto-bleaching of the reaction mixture has been reported (38), and to minimize this, the reaction mixtures were incubated at 30°C for 10 min before 190 μl of the reaction mixture was added to the microplate well containing 10 μl of 250 mM methanol or double-distilled H_2O (dd H_2O). Absorbance of the reaction mixture was followed at 600 nm in an Epoch2 microplate spectrophotometer (Biotek, Winooski, VT). Using this method, background activity in the absence of methanol was not detectible. The specific activity (SA) was calculated using a molar extinction coefficient at 600 nm (ϵ_{600}) for DCPIP of 21 $\text{mM}^{-1} \text{cm}^{-1}$ and a conversion factor of 0.667 to convert absorbance readings to a 1-cm path length.

RESULTS

XoxF and an unknown methanol oxidation system contribute to lanthanide-dependent methanol growth. To determine the requirements for the MxaFI, XoxF1, and XoxF2 MeDH enzymes during Ca- and lanthanide-dependent methanol growth, null mutations were constructed to generate *mxoF*, *xoxF1*, *xoxF2*, *xoxF1 xoxF2*, and *mxoF xoxF1 xoxF2* mutant strains. These strains were grown in methanol medium containing 20 μM Ca, 20 μM La, or both 20 μM Ca and 2 μM La (Fig. 1). The growth of these mutant strains in the absence of La was consistent with published data (22) that used hypho minimal medium (39) instead of MP medium (Fig. 1A). Strains lacking *mxoF* were unable to grow because the Ca-dependent MeDH had been eliminated, while strains lacking both *xoxF1* and *xoxF2* were unable to grow because XoxF is required for expression of *mxoF* (22). As reported by Nakagawa et al. (11), addition of La allowed wild-type growth of an *mxoF* mutant strain in methanol medium (Fig. 1B). Loss of *xoxF1* resulted in an increased lag of ~ 30 h and an increase in the doubling time (T_D) ($T_D = 5.5 \pm 0.2$ h [wild type]; $T_D = 8.6 \pm 0.9$ h [*xoxF1*]). Loss of *xoxF2* in addition to *xoxF1* significantly further increased the doubling time ($T_D = 19.1 \pm 0.8$ h), but surprisingly, the *xoxF1 xoxF2* mutant strain was able to grow. Growth was not eliminated in the *mxoF xoxF1 xoxF2* triple-mutant strain, suggesting that there is at least one additional, undiscovered enzyme or pathway that can oxidize methanol when La is present in the growth medium. When Ca and La were both added to the growth medium with the concentration of La 10-fold lower than that of Ca (Fig. 1C), the mutant phenotypes were identical to those when Ca was not added to the medium, indicating that La is dominant over Ca.

Methanol dehydrogenase activity in cell extracts is consistent with growth. To determine if MeDH activity correlates with growth, MeDH activity was measured in cell extracts prepared from strains grown in methanol medium containing 20 μM Ca with or without 2 μM La (Fig. 2). While a small increase in MeDH activity for the wild type was observed when cells were grown with La ($\text{SA}_{\text{Ca}} = 64 \pm 4 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$; $\text{SA}_{\text{La}} = 81 \pm 3 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$), the ~ 10 -fold increase in MeDH activity, as reported by Nakagawa et al., was not seen (11). Loss of *mxoF* alone or of *xoxF1* or *xoxF2* in addition to *mxoF* did not significantly decrease MeDH activity in La-grown cells, but loss of all known MeDHs together resulted in a 9-fold reduction in activity ($\text{SA}_{\text{mxoF xoxF1 xoxF2}} = 9 \pm 1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). This low level of MeDH activity is consistent with the

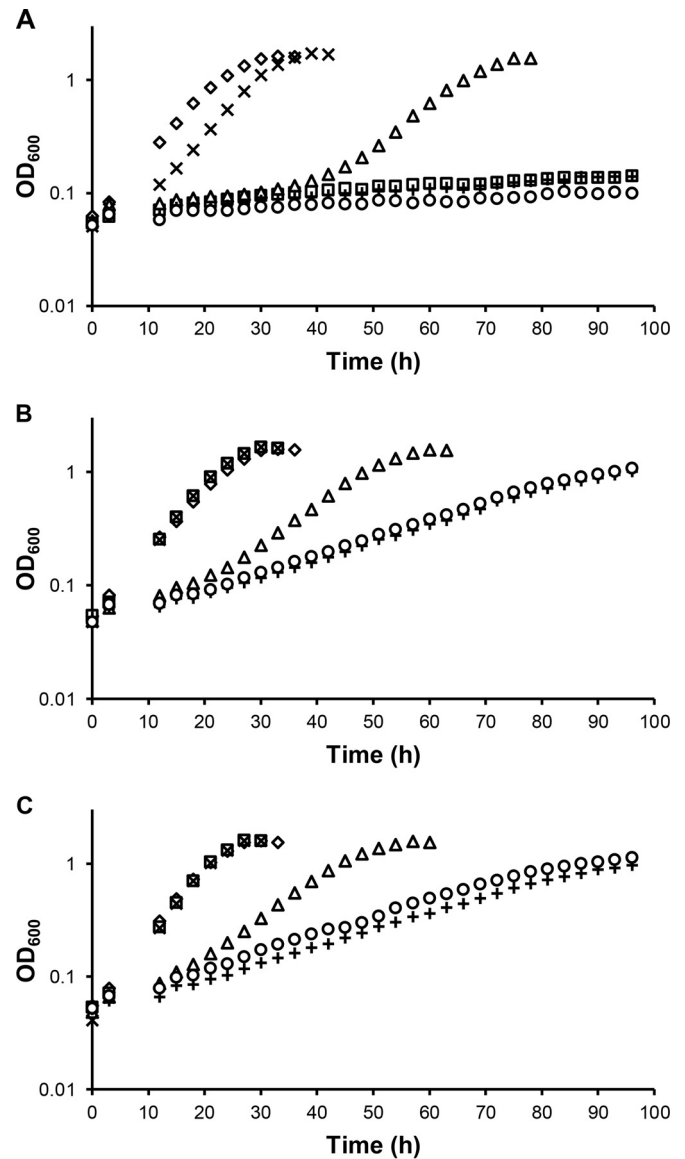


FIG 1 Growth of wild-type (\diamond), *mxoF* (\square), *xoxF1* (\triangle), *xoxF2* (\times), *xoxF1 xoxF2* ($+$), and *mxoF xoxF1 xoxF2* (\circ) strains in MP methanol medium containing 20 μM Ca (A), 20 μM La (B), or both 20 μM Ca and 2 μM La (C). The graphs show representative data obtained from three biological replicates. Variation in growth rates for all replicates was within 2%.

significantly increased doubling time for the MeDH triple-mutant strain in methanol medium containing La (Fig. 1C).

One micromolar La supports the maximum growth rate and optical density of an *mxoF* mutant strain during growth in methanol medium. To assess the concentrations of exogenous La that would support La-dependent methanol growth, the *mxoF* mutant strain was grown in methanol medium containing 20 μM Ca supplemented with La concentrations ranging from 0 to 20 μM (Fig. 3). The *mxoF* mutant strain was used to eliminate any contribution by the Ca-dependent MxaFI-MeDH. La-dependent methanol growth, albeit slow, was observed using La concentrations as low as 2.5 nM, while 1 μM La resulted in maximal growth and culture density.

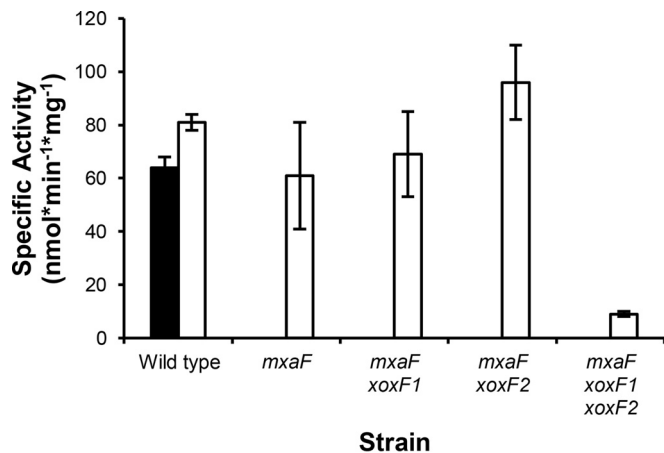


FIG 2 Methanol dehydrogenase activities in wild-type, *mxnF*, *mxnF xoxF1*, *mxnF xoxF2*, and *mxnF xoxF1 xoxF2* strains in MP methanol medium containing 20 μM Ca (black bar; wild type only) and both 20 μM Ca and 2 μM La (white bars). Average specific activities ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) were determined from two biological and six technical replicates. The error bars represent standard deviations from the means.

Multiple lanthanides support growth of the *mxnF* and *mxnF xoxF1 xoxF2* mutant strains. To investigate the abilities of different lanthanides to support methanol growth, 2 μM (each) La, Ce, Pr, Nd, Sm, Dy, Ho, Er, and Yb was added to methanol medium, and growth of the *mxnF* and *mxnF xoxF1 xoxF2* mutant strains was assessed (Fig. 4). Growth occurred with the first four lanthanides, La, Ce, Pr, and Nd, while 2 μM Sm allowed growth of the *mxnF* mutant strain at a significantly reduced rate ($T_D = 67.2 \pm 1.5$ h) (Fig. 4A). To determine if increased Sm concentrations would improve growth of the *mxnF* mutant strain, 20 μM Sm was also tested. In all three *mxnF* biological replicates, an increase in the growth rate occurred after 150 h, resulting in a doubling time of 18.3 ± 0.9 h. To ensure cultures were not contaminated, all the strains were streaked onto methanol media lacking or containing La. Growth of the *mxnF* mutant strains from Sm cultures did not occur on medium lacking La, but they grew like the wild type on medium containing La. While this confirmed that *mxnF* was still nonfunctional and contamination did not occur, we were not able to confirm or refute whether a second site suppressor mutation arose that specifically allowed increased Sm-dependent growth. Similarly, growth of the MeDH triple-mutant strain occurred with La, Ce, Pr, and Nd and occurred at a reduced rate with Sm. Intriguingly, Sm-dependent growth of the MeDH triple mutant is faster than with the *mxnF* mutant alone when 2 μM Sm is present ($T_D = 67.2 \pm 1.5$ h [*mxnF*]; $T_D = 18.0 \pm 0.9$ h [*mxnF xoxF1 xoxF2*]).

Expression from the *mxn* and *xox1* promoters is differentially regulated by La. It was reported by Nakagawa et al. that exogenous La does not increase expression from the *mxn* and *xox1* promoter regions and that regulation is likely to occur posttranslationally (11). However, when using transcriptional reporter fusions that contain significantly larger regions of DNA that extend into the first gene within the operon (1,304 bp versus 217 bp for the *mxn* promoter region; 1,351 bp versus 209 bp for the *xox1* promoter region), differential expression was seen, with expression from the *xox1* promoter upregulated in the presence of La and expression from the *mxn* promoter upregulated in the absence of La (Fig. 5A). It was previously shown that *xoxF* (but not

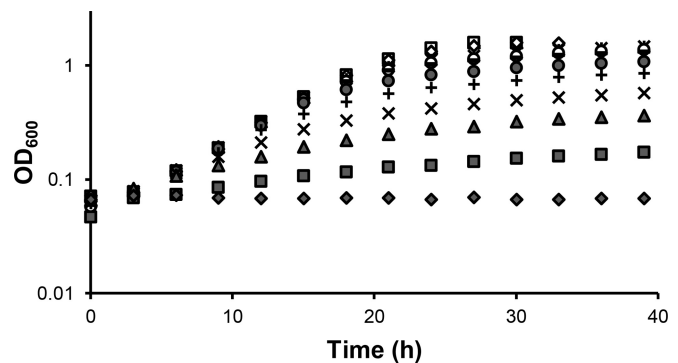


FIG 3 Growth of the *mxnF* mutant strain in MP methanol medium containing 20 μM Ca (\blacklozenge) and 20 μM Ca with increasing concentrations of La: 2.5 nM (\blacksquare), 5 nM (\blacktriangle), 10 nM (\times), 25 nM ($+$), 50 nM (\bullet), 100 nM ($-$), 250 nM (\circ), 500 nM (\times), 1 μM (\diamond), 2 μM (\square), and 20 μM (\triangle). The growth curve shows representative data from three biological replicates. Variation in growth rates between biological triplicates was within 1%.

mxnF) is required for expression of *mxnF* and for repression of *xoxF* in hypho minimal medium lacking La (22). These studies were repeated here to determine if expression from the *mxn* and *xox1* promoters behaves identically in the more robust MP medium (36) and to determine how loss of *mxnF* and *xoxF1* affects expression in medium containing La. As *mxnF* and *xoxF1* are required for methanol growth in the absence of lanthanides, succinate was added as an additional carbon source. These studies confirm that loss of *mxnF* does not significantly affect expression from the *mxn* and *xox1* promoter regions (Fig. 5B) but that *xoxF1* is required for expression from the *mxn* promoter and for repression of itself (Fig. 5C). When *xoxF1* is absent, expression from the *xox1* promoter in medium lacking La is similar to expression in the presence of La. Transcriptional reporter fusion studies were also conducted using the *xoxF2* promoter region; however, these assays were not sensitive enough to measure expression above background from the promoter, which has a low level of expression (39).

Expression from the *mxn* and *xox1* promoter regions in the presence of increasing concentrations of La. To determine how sensitive the *mxn* and *xox1* transcriptional reporter fusions are to the presence of exogenous La, cells were grown in the presence of increasing concentrations of La (0 to 20 μM). An increase in expression from the *xox1* promoter was seen using La concentrations as low as 2.5 nM, with expression reaching maximum at 250 nM (Fig. 6). Concentrations of La between 25 and 50 nM resulted in decreased expression from the *mxn* promoter, with full repression seen at 250 nM. Expression from both promoters with La concentrations above 250 nM (tested up to 20 μM) was nearly identical to the expression seen using 250 nM La (data not shown).

Addition of La, Ce, Pr, and Nd results in differential expression from the *mxn* and *xox1* promoter regions. To determine if lanthanide-dependent expression correlates with lanthanide-dependent methanol growth, different lanthanides were tested for the ability to repress expression from the *mxn* promoter and up-regulate expression from the *xox1* promoter. Similar to growth, differential expression was observed when 2 μM La, Ce, Pr, and Nd was present, and a small effect on expression was observed when Sm was present in the growth medium (Fig. 7).

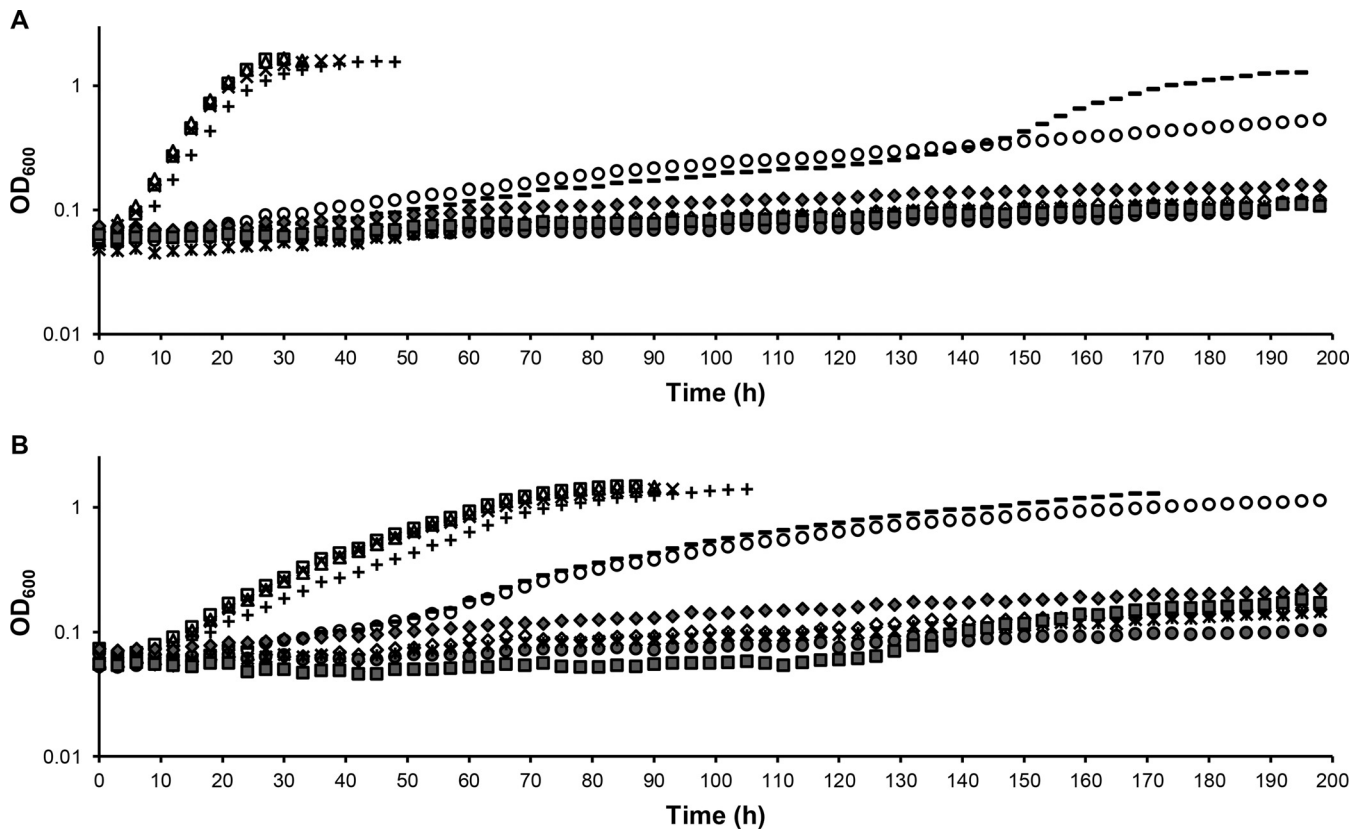


FIG 4 Growth of *mxoF* (A) and *mxoF xoxF1 xoxF2* (B) mutant strains in MP methanol medium containing 20 μM Ca (\diamond) and 20 μM Ca with 2 μM different lanthanides—La (\square), Ce (\triangle), Pr (\times), Nd ($+$), Dy (\bullet), Ho (\times), Er (\blacklozenge), and Yb (\blacksquare)—with the exception of Sm, tested at 2 μM (\circ) and 20 μM ($-$). The graphs show representative data from three biological replicates. Variation in growth rates between biological triplicates was within 2%.

DISCUSSION

Using MeDH mutant strains, we show that methanol-dependent growth of an *mxoF* mutant strain can occur if La, Ce, Pr, Nd, and, to a lesser extent, Sm are added to the growth medium and that *M. extorquens* AM1 preferentially uses lanthanides over Ca even when lanthanides are present at a 10-fold-lower concentration. It has been suggested that lanthanides are more efficient Lewis acids than Ca and may enhance the catalytic properties of MeDH (25). Additionally, initial characterization of two XoxF-type MeDHs demonstrated superior oxidation capacities, so that in one case, oxidation of methanol leads to direct production of formate instead of formaldehyde (8). Surprisingly, our growth studies reveal the existence of at least one additional, unknown lanthanide-dependent enzyme or pathway that can oxidize methanol. Once this new methanol oxidation system is revealed and biochemically characterized, it may become clearer why *M. extorquens* AM1 has multiple enzymes that carry out the same function. Enzyme redundancy in *M. extorquens* AM1 is a common occurrence. For example, there are at least four distinct formate dehydrogenases that oxidize formate to CO_2 (40, 41), four copies of the *zwf* gene encoding glucose-6-phosphate dehydrogenase, and five copies of a putative oxalate formate antiporter (21). The discovery of a novel non-XoxF lanthanide-dependent methanol oxidation system may have a significant impact on the field of environmental microbiology, helping to ascertain whether organisms previously classified as non-methylophs are, in fact, methylophic after all. It may be that

some organisms contain only this unknown methanol oxidation system to oxidize methanol for energy, if not for carbon.

The ability of only La, Ce, Pr, and Nd, and Sm to a lesser extent, to facilitate methylophic growth and differential expression of the Ca- and lanthanide-dependent MeDH genes could be explained by several distinct possibilities. It may be that the cofactor range for XoxF-MeDH and the unknown methanol oxidation system is limited by the ionic radius of the lanthanide. If the ionic radius is too large, it may not be inserted into XoxF. It is also possible that larger lanthanides could support XoxF function but are unable either to be transported into the cell or to be sensed by the regulatory network controlling *mxo* and *xoxI* expression. Detailed biochemical and molecular analyses are needed to distinguish between these possibilities.

Each of the above-mentioned possibilities could explain why increasing the Sm concentration 10-fold in the medium allows increased growth (Fig. 4). The ~ 150 -h lag seen for the *mxoF* mutant strain in the presence of Sm is intriguing. One possible explanation for this lag could be that Sm is poorly sensed and transported into the cell but allows high activity of the lanthanide-dependent MeDHs once inside. If this is true, then *xoxF1* would be expressed at a very low level, consistent with the expression data shown in Fig. 7, and it would take significant time to produce enough active XoxF to allow rapid growth. A second explanation for the increase in the growth rate of the *mxoF* mutant strain after 150 h is that a second site suppressor mutation arose

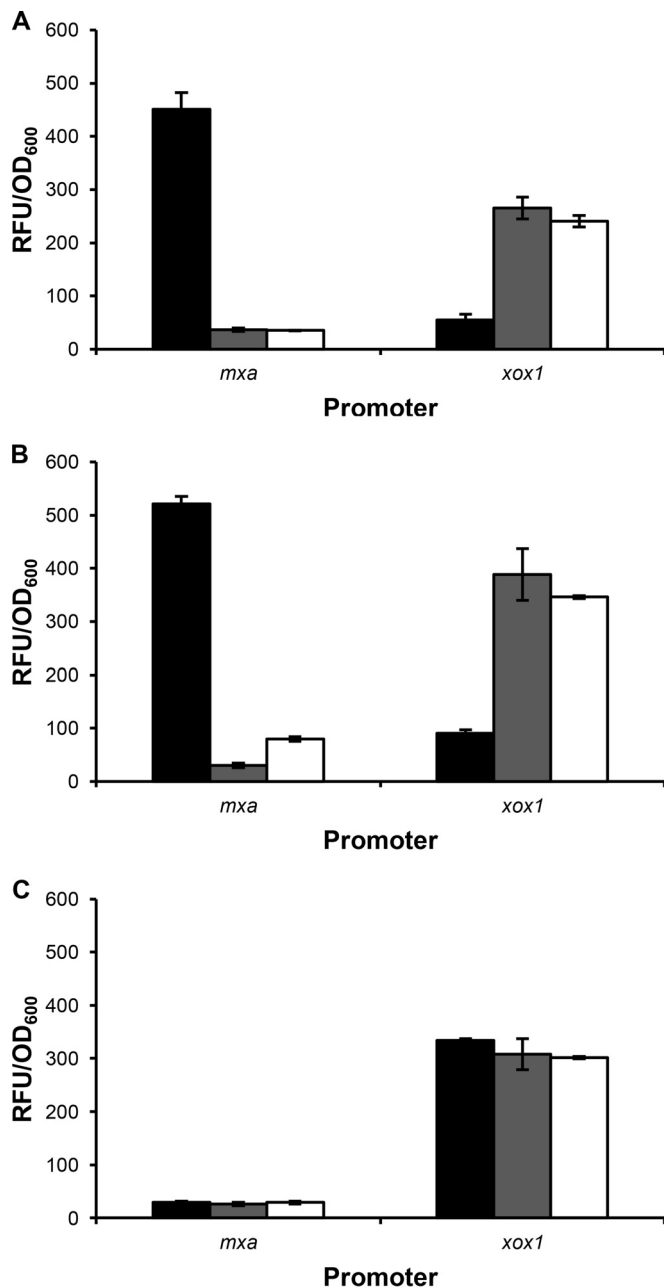


FIG 5 Expression from the *mx*a and *xox*1 promoters using Venus as a transcriptional reporter in wild-type (A), *mx*aF (B), and *xox*F1 *xox*F2 (C) strains. The strains were grown in MP succinate-methanol medium containing 20 μ M Ca (black bars), 20 μ M La (gray bars), or both 20 μ M Ca and 2 μ M La (white bars). The expression levels are reported as RFU per OD₆₀₀ unit. The background expression levels from the promoterless *venus* fusion vector in the wild-type, *mx*aF, and *xox*F1 *xox*F2 strains were 59 ± 6 RFU/OD₆₀₀, 90 ± 6 RFU/OD₆₀₀, and 82 ± 10 RFU/OD₆₀₀, respectively. The data represent the average RFU/OD₆₀₀ values from biological triplicates. The error bars show standard deviations from the means.

that resulted in, for example, upregulation of the unknown lanthanide-dependent MeDH or increased transport of Sm.

Curiously, growth of the *mx*aF *xox*F1 *xox*F2 triple-mutant strain is faster than that of the *mx*aF single-mutant strain in the presence of Sm (Fig. 4). It is intriguing to speculate that the un-

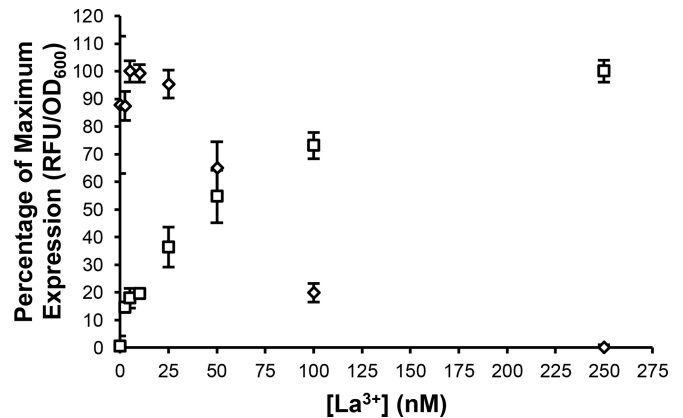


FIG 6 Expression from the *mx*a (\diamond) and *xox*1 (\square) promoters with increasing concentrations of La. Expression values are reported as the percentage of maximum expression of the *mx*a-*venus* and *xox*1-*venus* transcriptional fusions in the wild type grown in MP succinate-methanol medium containing both 20 μ M Ca and various concentrations of La. The data depict the average expression from biological triplicates with background expression levels from the promoterless *venus* fusion vector subtracted. The error bars represent standard deviations from the means.

known methanol oxidation system may be upregulated in the absence of *xox*F1. XoxF has been shown to be required for expression of the *mx*a genes and for repression of the *xox*1 genes (22) (Fig. 5) and may also be involved in regulation of the unknown system.

The MeDH activity and MeDH gene expression data presented here conflict with previously published data (11). Nakagawa et al. reported that exogenous La resulted in a 10-fold increase in MeDH activity. Additionally, the specific activities for MeDH in cell extracts were significantly different. This could be due in part to differences in strain backgrounds, how the cells were grown (the medium used and the growth phase of the cells isolated), and how the assays were conducted. When conducting MeDH assays using the protocol reported by Nakagawa et al., MeDH activity from the wild type grown in methanol medium with La was higher ($138 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ after subtracting background oxidation) than when activity was measured using our assay modifications ($81 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$); however, the rate of methanol-independent oxidation, or auto-bleaching, was $283 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ when methanol was added to the assay mixture. This high background activity was observed for all the strains. Additionally, MeDH activity in the *mx*aF *xox*F1 *xox*F2 triple-mutant strain grown in methanol medium containing La was not detectable when using the assay. By preincubating the reaction mixture for 10 min before substrate addition, background oxidation was eliminated and activity for the unknown methanol oxidation system could be detected. The activities reported here for Ca-dependent methanol growth are identical to the activities reported by Smejkalová et al. (42). Smejkalová et al. noted that wide discrepancies in MeDH activity have been published for years, with specific activities for the Ca-dependent MeDH ranging from 64 to $540 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, depending on the protocol used to measure MeDH activity.

The question remains as to how cells sense, acquire, and transport lanthanides so that the lanthanide-dependent MeDH enzymes, but not the Ca-dependent MeDH, are used when lanthanides are present. In the methanotrophic bacterium *M. trichosporium* OB3b, it was shown that the presence of lanthanides

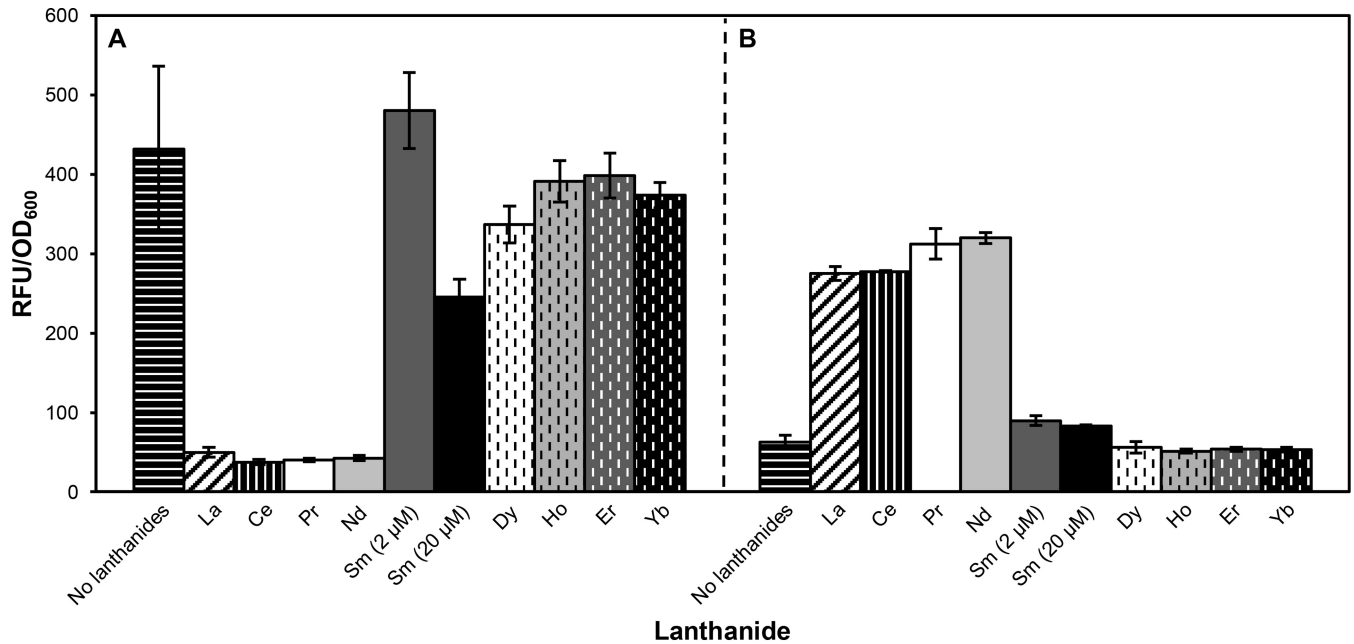


FIG 7 Expression from the *mx*a (A) and *xox*1 (B) promoters in the wild type grown in MP succinate-methanol medium containing 20 μ M Ca without (control) and with 2 μ M different lanthanides, with the exception of Sm (tested at 2 μ M and 20 μ M). The background expression level from the promoterless *venus* fusion vector was 58 ± 5 RFU/OD₆₀₀. The data were obtained from three biological replicates, and the error bars show standard deviations from the means.

upregulates expression of the *xoxF*-MeDH genes and represses expression of the *mx*aFI-MeDH genes. However, in *M. extorquens* AM1, it was reported that lanthanides do not affect expression of the *mx*a or *xox*1 genes and that regulation is likely to occur post-translationally (11). We show that in *M. extorquens* AM1, differential expression from the *mx*a and *xox*1 promoters is very sensitive to the presence of La, Ce, Pr, and Nd. Titration experiments with La showed that induction of expression from the *xox*1 promoter occurs with concentrations as low as 2.5 nM and reaches maximum expression with 250 nM La. Repression from the *mx*a promoter is seen when concentrations of >25 nM La are present, with full repression occurring with 250 nM La. As these reporter fusions are contained on a multicopy plasmid, these data likely do not reflect the exact concentrations required for chromosomal expression and repression from these promoters, but they do suggest that there may be a lanthanide concentration range in which both MxaFI and XoxF enzymes are expressed and used equally.

Our expression data refute the conclusions of Nakagawa et al. and provide a likely explanation for the discrepancy in the results due to the different lengths of the promoter regions used in these comparative studies (11). The promoter regions used in our studies are significantly larger and extend well into the *xoxF*1 and *mx*aF genes to include any possible repressor binding sites, while the Nakagawa et al. study used much shorter promoter regions that end at the start of the *xoxF*1 gene and 114 bp before the start of the *mx*aF gene. In the absence of lanthanides, it is known that the response regulator MxbM is required for repression of the *xox*1 operon (22). The lack of repression of the *xox*1 genes seen by Nakagawa et al. may be due to the lack of the repressor binding site on the region of DNA chosen for use in their study. Additionally, the level of expression seen from their *mx*a promoter region in the absence of lanthanides was low, even though the genes encoding

the Ca-dependent MeDH are among the most highly expressed in the cell (39). It is possible that the *mx*a promoter region used in this study lacks the binding site for a transcriptional activator. As direct binding by regulators required for *mx*a expression has never been demonstrated, it is difficult to determine if this explanation is correct or if these differences are due to differences in the media used and the strain backgrounds.

Taken together, these data expand on the regulatory hypothesis proposed by Skovran et al., where apo-XoxF may function as a cellular sensor of lanthanide presence in the periplasm, interacting with one or both of the sensor kinases MxcQ and MxbD (Fig. 8). When lanthanides are absent, MxcQ and/or MxbD could signal to the MxcE and/or MxbM response regulators to activate *mx*a expression and repress *xox*1 expression (22). When lanthanides are present, apo-XoxF may be converted to active XoxF, thereby decreasing the signal to MxcQ/MxbD, which would result in upregulation from the *xox*1 promoter and repression from the *mx*a promoter. As more is learned about how expression of the various MeDH genes is controlled, a more complete and accurate model can be described.

The discovery of a non-XoxF type of lanthanide-dependent MeDH is intriguing. This finding could greatly impact the environmental study of methylophony communities, as it is possible that organisms that have only this third, unknown system are disregarded as nonmethylophony. While it is clear that lanthanides differentially control the expression of the XoxF- and MxaFI-MeDHs, the regulatory network controlling expression of these MeDH genes is complex and poorly understood and has been investigated only in the absence of lanthanides (22). Once it is better understood how methylophony are able to sense and acquire lanthanides, it may be possible to engineer these bacteria as biorecycling and biomining agents to enhance yields from lanthanide mining and to recover these highly valued rare-earth ele-

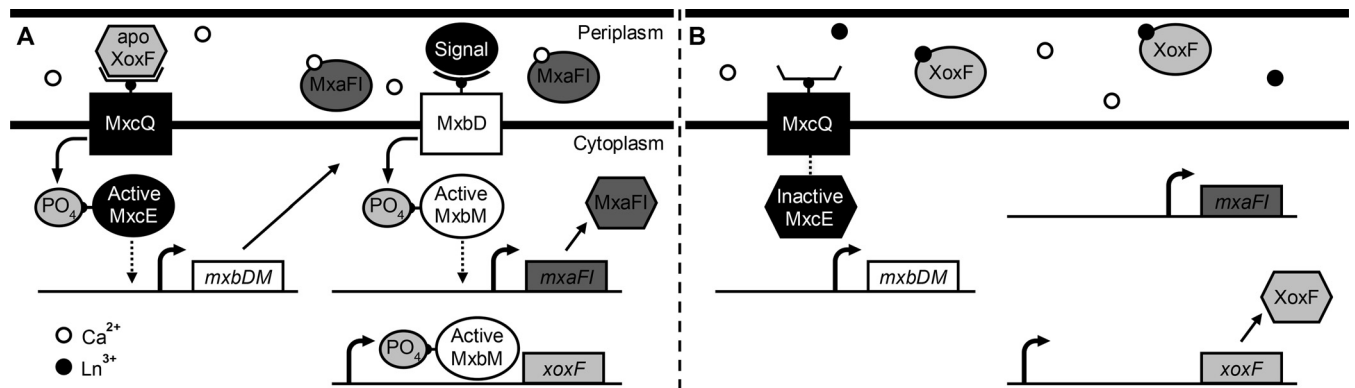


FIG 8 Hypothesis for how XoxF and lanthanides (Ln) may differentially regulate expression of the *mxs* and *xoxI* genes in the absence (A) and presence (B) of lanthanides. MxcQE and MxbDM are two-component systems required for expression of the *mxs* genes, though whether this requirement is direct or indirect has not been demonstrated. In this model, apo-XoxF acts as a sensor for lanthanide presence. (A) When lanthanides are absent, apo-XoxF activates expression of the *mxs* genes and represses expression of the *xoxI* genes as mediated through the two-component systems MxcQE and MxbDM. (B) When lanthanides are present, XoxF resumes its catalytic role as a lanthanide-dependent MeDH and is unavailable or not in the correct conformation to interact with the two-component systems, resulting in the repression of the *mxs* genes and activation of the *xoxI* genes.

ments from end-of-life products. As lanthanide-mining deposits continue to decrease while lanthanide demands continue to rise, the development of alternative mining and recovery strategies is an attractive prospect.

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