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XoxF Acts as the Predominant Methanol Dehydrogenase in the Type I Methanotroph *Methylomicrobium buryatense*

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ABSTRACT

Many methylotrophic taxa harbor two distinct methanol dehydrogenase (MDH) systems for oxidizing methanol to formaldehyde: the well-studied calcium-dependent MxaFI type and the more recently discovered lanthanide-containing XoxF type. MxaFI has traditionally been accepted as the major functional MDH in bacteria that contain both enzymes. However, in this study, we present evidence that, in a type I methanotroph, *Methylomicrobium buryatense*, XoxF is likely the primary functional MDH in the environment. The addition of lanthanides increases *xoxF* expression and greatly reduces *mxa* expression, even under conditions in which calcium concentrations are almost 100-fold higher than lanthanide concentrations. Mutations in genes encoding the MDH enzymes validate our finding that XoxF is the major functional MDH, as XoxF mutants grow more poorly than MxaFI mutants under unfavorable culturing conditions. In addition, mutant and transcriptional analyses demonstrate that the lanthanide-dependent MDH switch operating in methanotrophs is mediated in part by the orphan response regulator MxaB, whose gene transcription is itself lanthanide responsive.

IMPORTANCE

Aerobic methanotrophs, bacteria that oxidize methane for carbon and energy, require a methanol dehydrogenase enzyme to convert methanol into formaldehyde. The calcium-dependent enzyme MxaFI has been thought to primarily carry out methanol oxidation in methanotrophs. Recently, it was discovered that XoxF, a lanthanide-containing enzyme present in most methanotrophs, can also oxidize methanol. In a methanotroph with both MxaFI and XoxF, we demonstrate that lanthanides transcriptionally control genes encoding the two methanol dehydrogenases, in part by controlling expression of the response regulator MxaB. Lanthanides are abundant in the Earth's crust, and we demonstrate that micromolar amounts of lanthanides are sufficient to suppress MxaFI expression. Thus, we present evidence that XoxF acts as the predominant methanol dehydrogenase in a methanotroph.

An increasing surplus in the global methane budget exists due to human activity. The industrial use of microorganisms to convert methane into useful chemicals or biofuels represents one way to mitigate atmospheric methane (1, 2). Methanotrophs, or methane-oxidizing bacteria, utilize methane as their carbon and energy source and are prime candidates for the industrial bioconversion of methane (1). Renewed interest in the industrial use of methanotrophs has come about partially because of the discovery of rapidly growing strains and new tools for genetic manipulation, allowing for fast-paced metabolic engineering (3, 4). The success of metabolic engineering strategies in these methanotrophs depends upon a strong foundation of knowledge concerning the metabolic pathways that methanotrophs employ, both in the laboratory and in their natural environments, and an understanding of how various branches of metabolic pathways are regulated.

The majority of methanotrophs have two systems for oxidizing methane to methanol: the particulate methane monooxygenase (pMMO) is a copper-dependent enzyme, and the soluble methane monooxygenase (sMMO) is an iron-dependent enzyme with a broader substrate specificity (5, 6). The transcriptional expression of these two methane oxidation systems is regulated by the presence of copper. Expression of the genes encoding pMMO is increased in the presence of copper, and expression of the genes encoding sMMO is decreased under these conditions (7, 8).

The methanol generated from methane oxidation is further oxidized to formaldehyde by a methanol dehydrogenase (MDH) enzyme. In methanotrophs, as well as in nonmethanotrophic methylotrophs, the oxidation of methanol to formaldehyde was traditionally thought to be catalyzed primarily by a calcium-dependent MDH, MxaFI. A gene similar to the one encoding MxaF, named *xoxF*, has been identified in many methylotrophs (6), and it has been recently demonstrated that *xoxF* encodes an alternative MDH, named XoxF (9–15). Moreover, it has become evident that *xoxF* genes are more widespread in methylotrophs than *mxaFI* genes (6, 16). An additional function for XoxF, in controlling *mxa* gene expression in the model methylotroph *Methylobacterium extorquens* AM1, has also been demonstrated (17).

The traditional MxaFI-type MDH is much more well studied than XoxF. MxaFI is a pyrroloquinoline quinone (PQQ)-linked MDH and is located in the periplasm (18). In methylotrophs, the

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mxa operon typically consists of genes encoding the large and small subunits of MDH (*mxaF* and *mxaI*, respectively), the cytochrome *c* electron acceptor (*mxaG*), proteins for calcium insertion (*mxaACKL*), and other genes of unknown function that are required for a functional MDH (6, 19). Transcription of this operon in *M. extorquens* AM1 is controlled by at least two two-component systems, MxcQE and MxbDM, as well as by an orphan response regulator, MxaB (20–23).

XoxF enzymes have been isolated as active MDH enzymes in multiple methylotrophs when the cultivation medium was supplemented with lanthanides (13-15, 24). Growth in a medium supplemented with lanthanides has been demonstrated to increase the MDH activity of the methylotrophs *Methylobacterium radiotolerans*, *Bradyrhizobium* sp., and *M. extorquens* AM1, when XoxF acts as the dominant MDH (13–15). Lanthanides have been copurified with the PQQ-linked XoxF enzyme, indicating that the active XoxF enzyme contains alternative metals, in contrast to the calcium-dependent MxaFI MDH (13, 24). Lanthanides are stronger Lewis acids than calcium and are proposed to act as a strong Lewis acid in the XoxF enzyme, allowing the active carbons in PQQ to become stronger electrophiles and remove electrons from methanol (24). The discovery of XoxF functionality in the presence of lanthanides has allowed for the cultivation of a novel acidophilic methanotroph, *Methylacidiphilum fumariolicum* SolV (24), as well as for growth of methylotrophic bacteria cultured from the phyllosphere (25). Lanthanides belong to a group of elements known as the rare earth elements, but they are relatively abundant in the Earth's crust despite the group's name (25). Some rare earth elements are present at concentrations similar to those of typical industrial metals (26, 27).

More recently, it has been shown that lanthanides transcriptionally regulate the expression of genes encoding the two alternative MDH enzymes in multiple methylotrophic organisms (25, 28). In the type II methanotroph *Methylosinus trichosporium* OB3b, *mxaF* gene expression was reduced in the presence of cerium, while *xoxF* expression was increased (28). These cerium-dependent effects were attenuated by the presence of copper, which led the authors to hypothesize that the MxaFI MDH likely formed a supercomplex with pMMO in the periplasm.

In this study, we examined the role of XoxF in a type I methanotroph, *Methylomicrobium buryatense* 5GB1C. Type I methanotrophs are particularly attractive for industrial use, as they assimilate formaldehyde into biomass using a highly efficient variant of the ribulose monophosphate pathway (5, 29–31). Our results suggest that XoxF is not an accessory MDH but is the preferred methanol oxidation system in *M. buryatense* 5GB1C. We show that lanthanides increase *xoxF* expression and decrease *mxa* expression, even in the presence of excess calcium. Results from knockout mutations in the MDH genes indicate that each system is dispensable under specific environmental conditions, i.e., the presence versus the absence of lanthanides. Overall, XoxF likely dominates environmental MDH activity, as micromolar concentrations of lanthanides are sufficient to completely block *mxa* expression. We demonstrate that the lanthanide-mediated MDH switch is regulated in part by the response regulator MxaB.

MATERIALS AND METHODS

Strains and growth conditions. *M. buryatense* 5GB1C and its derivatives were grown in modified nitrate mineral salts medium (NMS2) as previously described (3). For growth without copper, the 500× high-purity trace elements solution was modified to contain 1.0 g of Na_2-EDTA/liter, 2.0 g of $FeSO_4 \cdot 7H_2O$/liter, 0.8 g of $ZnSO_4 \cdot 7H_2O$/liter, 0.03 g of $MnCl_2 \cdot 4H_2O$/liter, 0.03 g of H_3BO_3/liter, 0.2 g of $CoCl_2 \cdot 6H_2O$/liter, 0.02 g of $NiCl_2 \cdot 6H_2O$/liter, and 0.05 g of $Na_2MoO_4 \cdot 2H_2O$/liter. Supplements were added as follows: 2.5% (weight/volume) sucrose, 100 μg of hygromycin/ml, 50 μg of kanamycin/ml, 30 μg of zeocin/ml, 30 μM lanthanum chloride (unless a different concentration is specified) (Sigma-Aldrich), and 30 μM cerium chloride (Sigma-Aldrich). All culturing glassware for experiments performed without lanthanides or copper was acid washed overnight in 1 M hydrochloric acid before use. All strains used in this study are listed in Table S1 in the supplemental material.

Genetic manipulations. All gene knockout constructs were composed of assembled PCR products that were electroporated into *M. buryatense* 5GB1C, as described previously (4). Briefly, a construct containing the zeocin resistance gene flanked by two FLP recombination target (FRT) sites was assembled in the middle of ~800 bp of the flanking region for each target gene deletion. The zeocin resistance gene was amplified from the zeocin resistance-*sacB* cassette described by Yan et al. (4). The resulting constructs were electroporated into *M. buryatense* 5GB1C. The knockout mutants were selected for by growth on media containing zeocin, which selects for colonies having undergone homologous recombination in the desired region, and confirmed by sequencing. The confirmed knockout strains were unmarked by electroporation of a plasmid, pFC25, containing the flippase gene (*flp*) to induce a site-specific recombination between the two FRT sites, thereby deleting the zeocin resistance gene (4). The final knockout strains contained unmarked gene deletions, with a single FRT site remaining, and were cured of pFC25. Primers for knockout assembly are listed in Table S2 in the supplemental material.

Strain FC31 containing P_{mxaF}-*xylE* was obtained by conjugation of a pCM433-based suicide plasmid, pFC30, harboring the P_{mxaF}-*xylE* construct between genes METBUDRAFT_2794 and METBUDRAFT_2795, into *M. buryatense* 5GB1C (4, 32) (see Table S1 in the supplemental material). Sucrose counterselection was used to unmark the strain (3). The P_{mxaF} portion contained 300 bp of sequence upstream of the *mxaF* open reading frame. This sequence contained almost the entire intergenic region between the *mxaF* and *mxaB* open reading frames, except for the 18 bp immediately upstream of the *mxaB* open reading frame. The *xylE* gene was amplified from pCM130 (33). Conjugation was performed with *Escherichia coli* S17-1 λpir acting as the donor strain, as previously described (3). Construction of the plasmid was performed using Gibson assembly (34). Primers for Gibson assembly are listed in Table S2 in the supplemental material.

Construction of the complementation strains is described in the Supplemental Materials and Methods.

Catechol 2,3-dioxygenase activity assay. Strain FC31, harboring P_{mxaF}-*xylE*, was grown to stationary phase in media with different concentrations of supplemented lanthanum, as indicated below (see Fig. 1C). Whole-cell quantitative catechol 2,3-dioxygenase (XylE) activity assays were performed using a protocol adapted from Ali and Murrell (35). Cells were centrifuged, and cell pellets were resuspended to an optical density at 600 nm (OD_{600}) of 0.5 in 50 mM Tris-HCl (pH 7.5). A catechol solution in 50 mM Tris-HCl (pH 7.5) was added to the cells to a final concentration of 1 mM (100 μl of total volume). Cells were assayed in a 96-well plate for catechol 2,3-dioxygenase activity by monitoring the absorbance at 375 nm to assay for the production of 2-hydroxymuconate semialdehyde. XylE activity was expressed in milliunits per minute per milligram of protein. Whole-cell XylE assays were performed successfully in other bacterial strains (36, 37).

RNA isolation. Cells were grown to early stationary phase for harvesting RNA. A 1:10 volume of stop solution (5% buffer-saturated phenol in ethanol) was added to cells prior to harvesting the cells by centrifugation. Cells were resuspended in RNA extraction buffer (1:3 ratio of 5% cetrimonium bromide in 2.5 M NaCl to 0.1 M phosphate buffer [pH 5.8]) and lysed by bead beating with 0.1-mm zirconia-silica beads (Biospec Products) in 50% phenol–chloroform–isoamyl alcohol (at a 25:24:1 ratio),

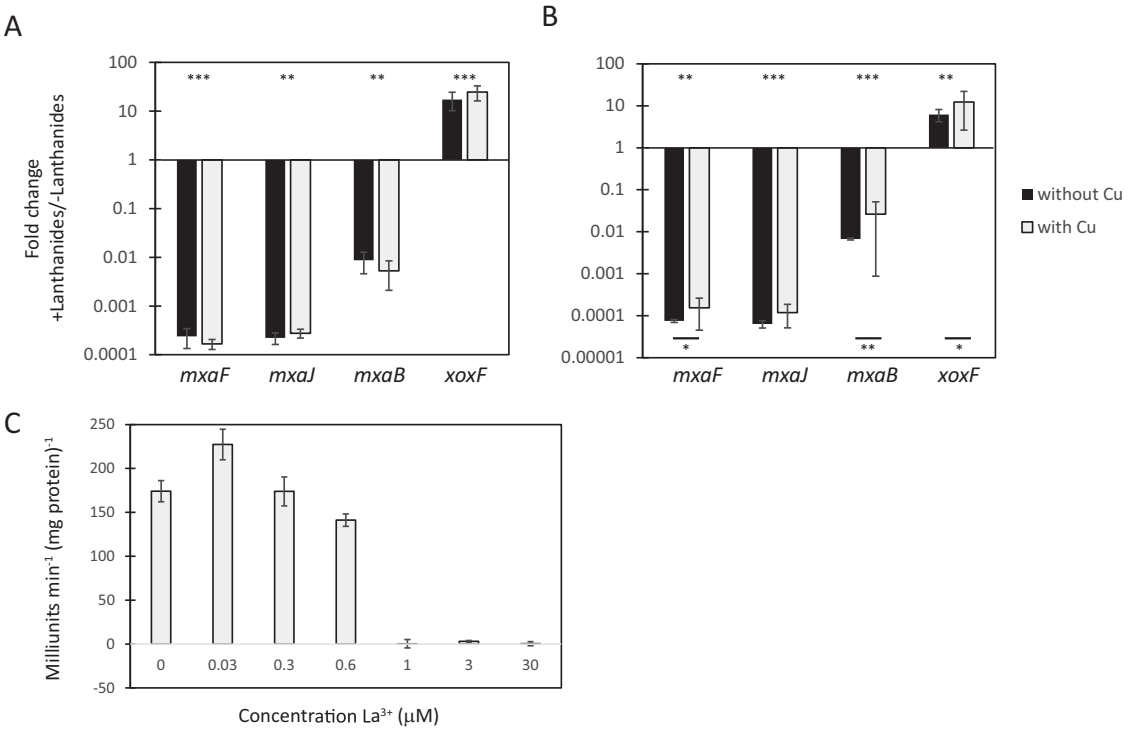

FIG 1 Lanthanides regulate *mxa* and *xoxF* genes divergently at the transcriptional level. Real-time qRT-PCR was performed on RNA harvested from *M. buryatense* 5GB1C cells grown in the presence or absence of supplemental 30 μM lanthanum (A) or 30 μM cerium (B), with and without copper. The values shown represent the fold change in *mxa* and *xoxF* gene expression from wild-type *M. buryatense* 5GB1C cells grown with lanthanides compared to wild-type *M. buryatense* 5GB1C cells grown without lanthanides. All C_T values were normalized to 16S rRNA. Multiple-way analysis of variance (ANOVA) was performed to determine significance of changes in gene expression levels (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Asterisks above the *x* axis indicate significance between with- and without-lanthanide conditions. Asterisks below the *x* axis indicate significance between the two data points connected by the bar. (C) Whole-cell catechol 2,3-dioxygenase activity from the *mxaF* promoter reporter strain FC31 (METBUDRAFT_2794::P_{mxaF}-*xylE*) grown in the indicated concentrations of supplemental lanthanum. Data represent means from three replicates ± standard deviations.

0.5% sodium dodecyl sulfate, and 0.5% *N*-lauroylsarcosine sodium salt. After centrifugation, the aqueous layer was harvested and mixed with an equal volume of chloroform-isoamyl alcohol (24:1 ratio). The aqueous layer was harvested again, and RNA was precipitated with 150 mM sodium acetate, 1.5 mM $MgCl_2$, and 50% isopropanol (all final concentrations) overnight at −80°C.

The precipitated RNA was harvested by centrifugation and treated with DNase I (Life Technologies) before purification with the RNeasy minikit and RNase-free DNase (Qiagen). We ensured that the harvested RNA was DNA free by using iScript reverse transcription supermix (Bio-Rad) with and without reverse transcriptase.

Real-time qRT-PCR assays. cDNA was generated using 100 to 500 ng of isolated RNA as the template with the SensiFast cDNA synthesis kit (Bioline). PCRs consisted of the following: 400 μM primers, SensiFast SYBR No-Rox kit (Bioline), cDNA, and double-distilled H_2O up to 10 μl of volume. The PCR mixtures were placed into LightCycler capillaries (Roche Diagnostics), and reactions were run using a LightCycler 2.0 (Roche Diagnostics). Threshold cycle (C_T) values were determined using LightCycler software, version 3.5 (Roche), and all gene expression values were normalized to 16S rRNA C_T values. All primers used for real-time quantitative reverse transcription-PCR (qRT-PCR) are listed in Table S2 in the supplemental material.

RESULTS

Lanthanides mediate the MDH switch in *M. buryatense* 5GB1C. We first determined whether lanthanides act as a switch between the two MDH enzymes, XoxF and MxaFI, in a type I methanotroph. We employed *M. buryatense* strain 5GB1C as our model type I methanotroph, as it is genetically tractable, has a rapid doubling time, and is an industrially promising methanotroph (3, 4). In *M. buryatense* 5GB1C, only one *xoxF* gene is present in the genome, and XoxF and MxaF share 49% identity at the amino acid level. We tested whether two lanthanides, lanthanum and cerium, regulate the expression of the *mxa* operon (consisting of genes *mxaF* to *mxaL*) and the *xoxF* gene. Real-time qRT-PCR was performed to measure relative transcript abundances using RNA isolated from *M. buryatense* 5GB1C cultures grown in the presence or absence of supplemental lanthanides. As shown in Fig. 1A and B, the transcription of two genes in the *mxa* operon, *mxaF* and *mxaJ*, was significantly reduced (3,700- to 15,000-fold decrease) when grown in normal growth medium containing calcium in the presence of cerium or lanthanum. Conversely, transcription of the *xoxF* gene was induced 6- to 25-fold in the presence of lanthanides (Fig. 1A and B). Together, these results suggest that lanthanides control a transcriptional MDH switch, with the MxaFI MDH operating in the absence of lanthanides and XoxF acting as the predominant MDH when lanthanides are present.

The effect of lanthanides on *mxa* gene expression was almost completely attenuated in the presence of copper in *M. trichosporium* OB3b, a type II methanotroph (28). To determine if a similar phenomenon occurred in *M. buryatense* 5GB1C, the real-time qRT-PCR experiments were performed on cells grown in the absence of copper, a condition which has been shown to induce

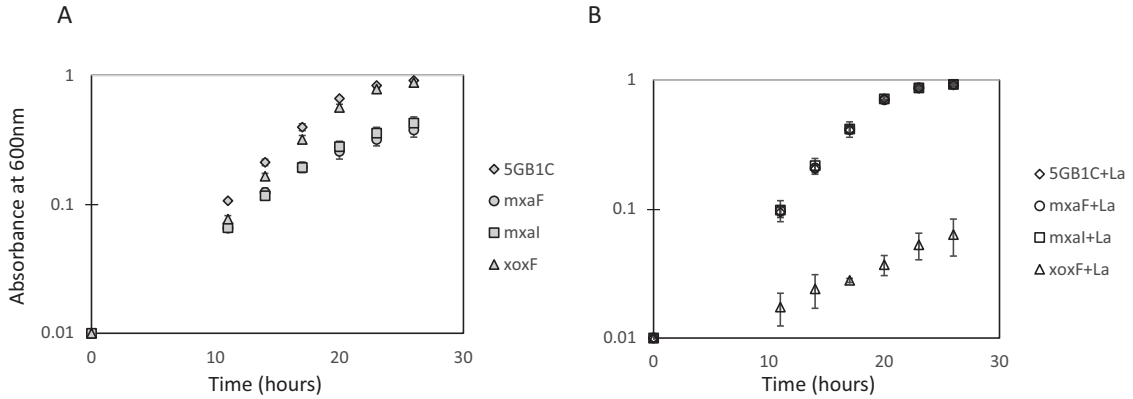

FIG 2 Analysis of MDH (*mxaF*, *mxaI*, and *xoxF*) mutants. Growth curves for MDH mutant strains and wild-type *M. buryatense* 5GB1C grown without (A) and with (B) supplemental 30 μM lanthanum (La). Data represent means from three replicates ± standard deviations.

sMMO activity (4). The absence of copper, and therefore the presence of sMMO expression, did not significantly modify *mxa* or *xoxF* expression in experiments with lanthanum (Fig. 1A). Copper slightly attenuated the effect of cerium on *mxaF* expression and slightly enhanced the influence of cerium on *xoxF* expression (Fig. 1B), but these copper-mediated effects were small compared to the effect of adding cerium, in contrast to the results reported for a type II methanotroph (28). Our results suggest that in *M. buryatense* 5GB1C, the choice between the two MDH enzymes is independent of the presence of copper. Since no significant effects were observed with copper when studying the lanthanum-dependent changes in *mxa* operon and *xoxF* expression, only lanthanum was used in subsequent experiments.

The decrease of 3 to 4 orders of magnitude in *mxa* expression by lanthanides in the presence of calcium suggests that XoxF may be the dominant MDH in environmental settings, where lanthanides are relatively abundant (26, 27, 38). To test this hypothesis in a laboratory setting, an *xylE* reporter gene was fused to the promoter of *mxaF* to create an *M. buryatense* 5GB1C strain with P_{mxaF}-*xylE* integrated into the chromosome at a region known to be transcriptionally silent (4). The resulting P_{mxaF} reporter strain was grown in media supplemented with different concentrations of lanthanides, and catechol 2,3-dioxygenase (XylE) activity was assayed. As little as 1 μM supplemented lanthanum was sufficient to completely repress P_{mxaF}, indicating that minute amounts of lanthanides in the environment would favor the utilization of XoxF over MxaFI (Fig. 1C).

MDH enzyme mutants display lanthanum-dependent growth defects. The differential regulation of the *mxa* operon and *xoxF* by lanthanides informed our construction of mutations in genes encoding the two MDH enzymes. A knockout mutant of *xoxF* was obtained by selecting for zeocin-resistant colonies in the absence of lanthanides. Conversely, knockout mutants in *mxaF* and *mxaI*, genes that encode the large and small subunits of the Mxa MDH, respectively, were selected on zeocin-containing media in the presence of lanthanum. The final versions of these mutants were unmarked, as described in Materials and Methods. Growth of these mutants was tested in the presence or absence of supplemental lanthanum. The Δ*xoxF* mutant displayed a wild-type growth rate in the absence of lanthanum, when the MxaFI enzyme was maximally expressed. However, we observed a marked growth defect (about a 70% decrease in growth rate) when Δ*xoxF* was grown in the presence of lanthanum, a condition under which *mxa* expression was significantly reduced (Fig. 2; Table 1). Likewise, the Δ*mxaF* and Δ*mxaI* mutants grew as well as wild-type *M. buryatense* 5GB1C did in the presence of lanthanum, when XoxF was maximally expressed (Fig. 2B) but displayed about a 20% decreased growth rate in the absence of supplemental lanthanum, when XoxF expression decreased by an order of magnitude (Fig. 2A; Table 1). Complementation of all three MDH mutants restored growth rates to wild-type levels, regardless of the presence or absence of lanthanum in the cultivation medium (see Table S3 in the supplemental material). These growth rate results are in line with the real-time qRT-PCR results presented above and further support the hypothesis that the MxaFI enzyme is required for MDH activity in the absence of lanthanides and that XoxF is required for MDH activity in the presence of lanthanides.

MxaB partially mediates the lanthanide switch. The *mxa* operon expression is known to be activated by at least two two-component systems, MxcQE and MxbDM, and an orphan response regulator, MxaB, in *M. extorquens* AM1 (20–23). The *M. buryatense* genome lacks homologs of genes encoding MxcQE or MxbDM, but a homolog of the *mxaB* gene is located upstream of the *mxa* operon and is divergently transcribed (39). Real-time qRT-PCR was performed to determine whether *mxaB* itself was regulated by lanthanides. Similar to gene expression results with *mxaF* and *mxaJ*, *mxaB* expression was significantly reduced in the presence of lanthanides (38- to 189-fold decrease) (Fig. 1A and B). This decrease in transcript abundance was independent of the presence of copper when lanthanum was present but was slightly

TABLE 1 Doubling times of MDH mutants

Strain	Doubling time[a] (h)	
	Without La^{3+}	With La^{3+}
WT 5GB1C[b]	3.17 ± 0.13	2.84 ± 0.15
Δ*mxaF* mutant	3.87 ± 0.45	2.88 ± 0.24
Δ*mxaI* mutant	3.87 ± 0.16	2.86 ± 0.12
Δ*xoxF* mutant	2.93 ± 0.07	10.3 ± 6.6

[a] Doubling times represent the means for three technical replicates with standard deviations. Doubling times were calculated from three time points during the exponential phase of growth.
[b] WT, wild-type *Methylomicrobium buryatense*.

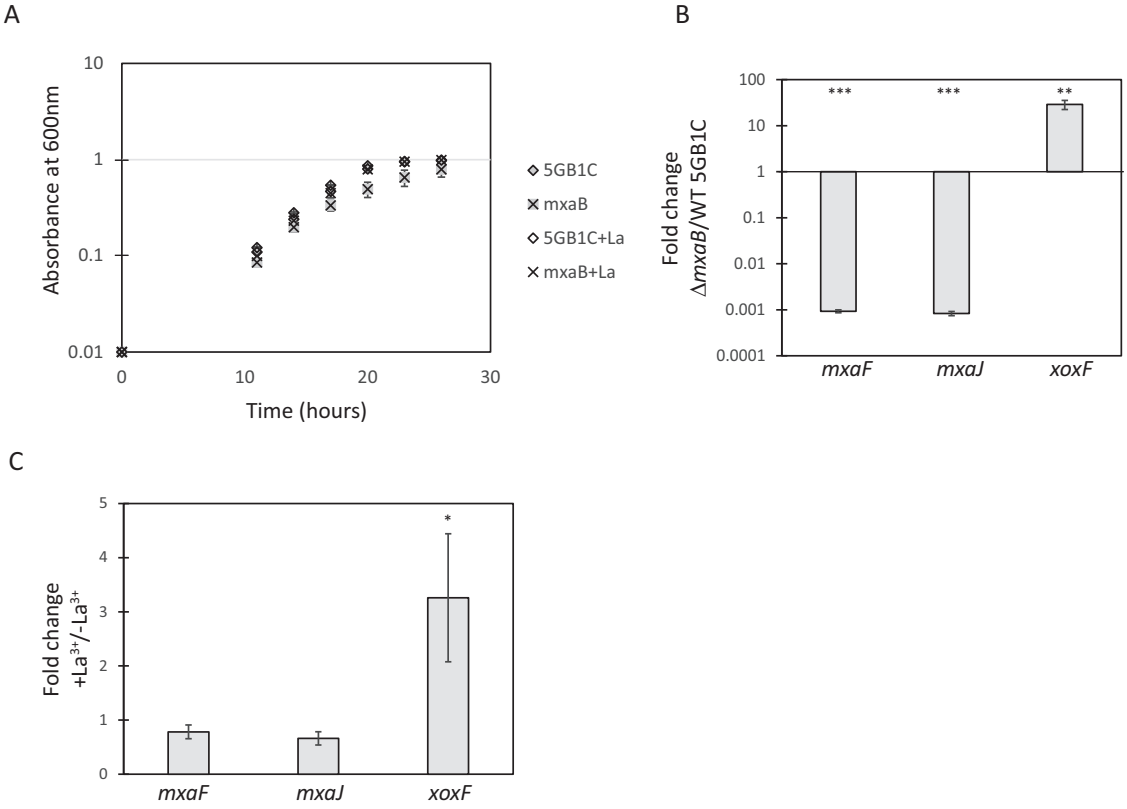

FIG 3 Lanthanide switch is mediated in part by MxaB. (A) Growth curves for the Δ*mxaB* mutant and for wild-type *M. buryatense* 5GB1C grown without and with supplemental 30 μM lanthanum (La). (B) Real-time qRT-PCR was performed on RNA harvested from the Δ*mxaB* mutant and wild-type *M. buryatense* 5GB1C cells grown in the absence of lanthanum. Results shown represent the fold change in gene expression in the Δ*mxaB* mutant compared to wild-type *M. buryatense* 5GB1C grown without lanthanum. (C) Real-time qRT-PCR was performed on RNA harvested from the Δ*mxaB* mutant grown with and without supplemental 30 μM lanthanum. Results shown represent the fold change in gene expression in Δ*mxaB* cells grown with lanthanum compared to gene expression in Δ*mxaB* grown without lanthanum. Unpaired *t* tests were used to determine significance (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$) between gene expression levels in panels B and C. Data represent means from three replicates ± standard deviations.

attenuated by copper when cerium was added to the cultivation medium (Fig. 1A and B).

We next investigated whether MxaB was involved in regulating *mxa* operon transcription in *M. buryatense* 5GB1C by creating a deletion mutation in *mxaB*. The Δ*mxaB* mutant grew at a similar rate to wild-type *M. buryatense* 5GB1C in the presence of lanthanum, but a slight growth defect was observed in the absence of lanthanum (Fig. 3A; Table 2). This phenotype is similar to the phenotypes of the Δ*mxaF* and Δ*mxaI* strains, indicating that MxaB may be required for obtaining a functional MxaFI enzyme. Real-time qRT-PCR was performed to determine MDH gene expression levels in the Δ*mxaB* mutant.

TABLE 2 Doubling times of Δ*mxaB* and Δ*xoxFS* mutants

Strain	Doubling time[a] (h)	
	Without La^{3+}	With La^{3+}
WT 5GB1C[b]	2.85 ± 0.18	2.79 ± 0.02
Δ*mxaB* mutant	3.07 ± 0.19	2.71 ± 0.08
Δ*xoxFS* mutant	2.71 ± 0.13	2.77 ± 0.03

[a] Doubling times represent the means for three technical replicates with standard deviations. Doubling times were calculated from three time points during the exponential phase of growth.
[b] WT, wild-type *Methylomicrobium buryatense*.

Compared to wild-type *M. buryatense* 5GB1C, when the Δ*mxaB* mutant was grown in the absence of lanthanum, *mxaF* and *mxaJ* expression levels were reduced by 1,000- and 1,200-fold, respectively (Fig. 3B). Accordingly, Δ*mxaB* cells harvested during growth with or without lanthanum had similar levels of *mxaF* and *mxaJ* expression (Fig. 3C). Together, these results indicate that *mxaB* expression is regulated by lanthanum and that its gene product activates *mxa* expression.

In *M. extorquens* AM1, the two-component system MxbDM acts to increase *mxa* expression while also decreasing *xoxF* expression (17). We found that MxaB functions similarly in *M. buryatense* 5GB1C by activating transcription of *mxa* genes and decreasing *xoxF* transcription. In the Δ*mxaB* strain, *xoxF* expression was enhanced 29-fold compared to the wild-type *M. buryatense* 5GB1C strain when both strains were grown in the absence of lanthanum (Fig. 3B). Transcription of the *xoxF* gene still exhibited a significant lanthanum-dependent increase in the Δ*mxaB* strain. However, this effect was attenuated in the Δ*mxaB* strain (3-fold increase) compared to the wild-type strain (~20-fold increase) (Fig. 1A and 3C).

Complementation of the Δ*mxaB* mutant restored the growth rate to wild-type levels, regardless of the presence or absence of lanthanum in the cultivation medium (see Table S3 in the supplemental material). The complemented strain also

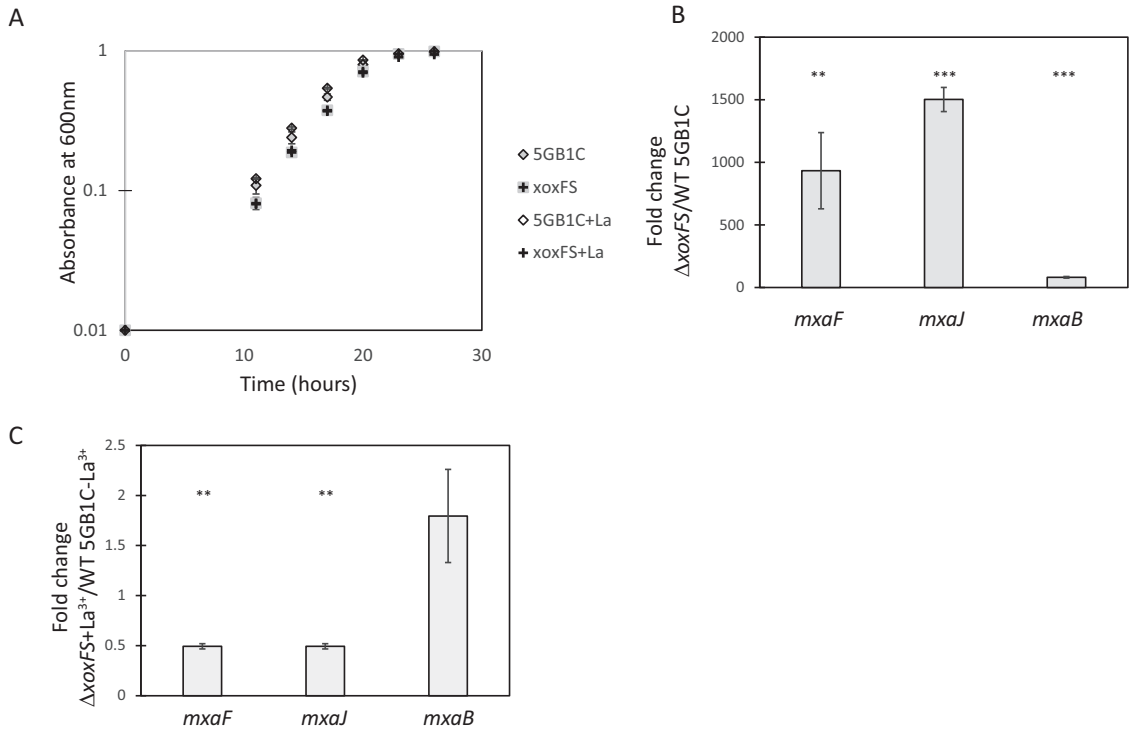

FIG 4 Suppressor mutation in Δ*xoxFS* mutant permits *mxa* expression in the presence of lanthanum (La). (A) Growth curves for Δ*xoxFS* strain and wild-type *M. buryatense* 5GB1C grown without and with supplemental 30 μM lanthanum. (B) Real-time qRT-PCR was performed on RNA harvested from the Δ*xoxFS* suppressor strain and wild-type *M. buryatense* 5GB1C grown in the presence of 30 μM supplemental lanthanum. Results represent the fold change in *mxa* gene expression in the Δ*xoxFS* strain compared to wild-type *M. buryatense* 5GB1C grown with lanthanum. (C) Real-time qRT-PCR was performed on RNA harvested from the Δ*xoxFS* suppressor strain grown in the presence of 30 μM supplemental lanthanum and wild-type *M. buryatense* 5GB1C grown without lanthanum. Results represent the fold change in *mxa* gene expression in Δ*xoxFS* grown with lanthanum compared to wild-type *M. buryatense* 5GB1C grown without lanthanum. Unpaired *t* tests were used to determine significance (***, $P < 0.001$) between gene expression levels in panels B and C. Data represent means from three replicates ± standard deviations.

restored wild-type lanthanide-mediated regulation of *mxaF*, *mxaJ*, and *xoxF* gene transcription (see Fig. S1 in the supplemental material).

Suppressors of the Δ*xoxF* mutant phenotype increase *mxa* expression in the presence of lanthanum. To determine whether additional components might be present that function in the lanthanide-mediated MDH switch, spontaneous mutants that suppressed the Δ*xoxF* mutant growth phenotype were isolated, as follows. We noted that, when grown for prolonged periods of time with lanthanum, many Δ*xoxF* cultures exhibited improved growth. Single colonies were isolated from these cultures and retested for growth in media supplemented with lanthanum. These isolates were grown in the absence of lanthanum before transfer to media supplemented with lanthanum to ensure that adaptation was not responsible for the improved growth. Six of eight of these isolates maintained robust growth in the presence of lanthanum, and one isolate was selected for further study. The Δ*xoxF* suppressor strain, named Δ*xoxFS*, displayed a wild-type growth rate regardless of the presence or absence of lanthanum (Fig. 4A; Table 2). This is in contrast with the results described above for the original Δ*xoxF* strain, which displayed a marked growth defect in the presence of lanthanum (Fig. 2B; Table 1).

We postulated that the Δ*xoxFS* variant was able to grow in the presence of lanthanum because the switch to reduce *mxa* expression under these conditions was nonfunctional in this strain. Indeed, *mxaF* and *mxaJ* expression levels were increased 900-fold and 1,500-fold, respectively, in the Δ*xoxFS* strain compared to wild-type *M. buryatense* 5GB1C when grown with lanthanum (Fig. 4B). This resulted in a comparable level of *mxaF* and *mxaJ* transcripts when the Δ*xoxFS* strain grown with lanthanum was compared to wild-type *M. buryatense* 5GB1C grown in the absence of lanthanum (Fig. 4C). We found that *mxaB* expression in the Δ*xoxFS* strain followed a similar pattern to that of the *mxaF* and *mxaJ* genes (Fig. 4B and C). The MxaB regulator present in the Δ*xoxFS* strain grown with lanthanum may be increasing *mxaF* to *mxaL* operon expression under these conditions. Together, the above results indicate that the lanthanide-mediated MDH switch is broken in the Δ*xoxFS* strain, permitting *mxa* expression in the presence of lanthanides.

We sought to identify the causal mutation in Δ*xoxFS* that allowed for lanthanide-independent expression of the *mxa* genes. The obvious candidates were (i) mutations in the shared promoter region of *mxaB* and *mxaF* that rendered the promoters unresponsive to lanthanide or (ii) mutations in the *mxaB* gene that allowed its product to be constitutively active. However, both of these regions had wild-type sequences in the Δ*xoxFS* strain (data not shown). These results suggest that there are additional control elements acting in the regulatory pathway upstream of MxaB and that these regulators function in a lanthanum-responsive manner to control *mxa* and *xoxF* transcription.

DISCUSSION

In this study, we demonstrated the existence of a lanthanide-mediated switch controlling the transcriptional expression of genes encoding two MDHs, the *xoxF* gene and the *mxa* operon, in a type I methanotroph. Similar results have been shown previously in the type II methanotroph *M. trichosporium* OB3b (28). However, in contrast to the results with *M. trichosporium* OB3b, copper does not significantly affect this switch, and lanthanides are the dominant factor determining which MDH is expressed, even in the presence of excess calcium. It has been suggested that, in *M. trichosporium* OB3b, XoxF can only serve as the MDH under conditions in which sMMO is expressed and that growth with pMMO would require MxaFI (28). However, in the type I methanotroph *M. buryatense* 5GB1C, it is clear that either MDH can support growth when either pMMO or sMMO is expressed. If an MDH forms a supercomplex with the pMMO, as has been suggested (28, 40), both MxaFI and XoxF must be able to form such a complex.

In the presence of 95 μM calcium in the cultivation medium, as little as 1 μM supplemental lanthanum was sufficient to completely abolish *mxa* gene transcription (Fig. 1C), showing that even with a major imbalance in the two metals, XoxF is the preferred MDH. Many methanotrophs, including *M. buryatense* isolates, have been isolated from sediment samples. In freshwater sediment samples collected from standard sediment collections, lanthanum concentrations have been reported to be around 42 μg lanthanum per g sediment (38). Lanthanum is largely insoluble under most environmental conditions; however, the sedimentary lanthanum concentrations found are approximately 300 times the concentration of lanthanum shown to inhibit P_{mxaF} transcription (0.14 μg lanthanum per g cultivation medium) (Fig. 1C). Therefore, the lanthanide concentrations found in the environment are likely sufficient to repress *mxa* transcription, and XoxF is likely the major functional MDH in *M. buryatense* in the environment. Metaproteomic analyses of environmental samples from the phyllosphere have shown that XoxF is expressed in naturally occurring methylotrophs that also encode *mxaFI* in their genomes (41, 42).

The growth defect of the Δ*xoxF* mutant cultured in the presence of lanthanum was much more pronounced than the growth defects exhibited by the *mxa* mutants (Δ*mxaF*, Δ*mxaI*, and Δ*mxaB*) cultured in the absence of supplemental lanthanum (Tables 1 and 2). From preliminary RNA sequencing results, we still observed a moderate number of *xoxF* transcript reads in cells grown without lanthanum (D. A. C. Beck, F. Chu, A. Gilman, and M. E. Lidstrom, unpublished results). However, we know that the *mxa* operon expression is completely suppressed in the presence of 1 μM lanthanum (Fig. 1C). Therefore, it is likely that XoxF is still being produced, albeit at significantly reduced levels, in the absence of supplemental lanthanum. Moreover, the Δ*mxaB* mutant had even more *xoxF* transcripts than wild-type cells in the absence of lanthanum (Fig. 3B) and, correspondingly, had a higher growth rate than the Δ*mxaF* or Δ*mxaI* mutants (Tables 1 and 2). In this study, all glassware was acid washed prior to any experiments performed without lanthanides, and all medium components were of the highest purity available. However, lanthanides are a component of some types of glass, and there may have been minute amounts present from this source or from contaminants in the media that allowed some XoxF function and therefore some growth in the Δ*mxa* mutants (27). Alternatively, it is possible that another metal present, such as Ca^{2+}, was able to partially support XoxF activity in the absence of lanthanum. Our results demonstrated that the Δ*xoxF* mutant had a more severe growth defect when grown in the presence of lanthanum than Δ*mxa* mutants when grown in the absence of lanthanum, and these results further support the conclusion that XoxF is the major functional MDH in *M. buryatense*.

The lanthanide MDH switch is mediated in part by the orphan response regulator MxaB, which has been shown to increase expression of the *mxa* operon in other methylotrophs (21, 22). No histidine kinase has been discovered to be responsible for MxaB phosphorylation, but we hypothesize the presence of a lanthanide-responsive histidine kinase that regulates MxaB activity. Lanthanides can be transported into the periplasm of Gram-negative cells to activate a regulatory cascade, as an engineered periplasmic histidine kinase has been shown to be responsive to lanthanides in *Escherichia coli* (43).

We hypothesize that additional regulatory genes are involved in the lanthanide-mediated MDH switch. In the Δ*mxaB* mutant, the *xoxF* gene is still partially induced by lanthanum, indicating that there is an additional factor controlling its transcription. Moreover, the Δ*xoxFS* mutant, which bears a broken lanthanide MDH switch, does not harbor mutations in the *mxa* operon promoter or the MxaB coding region. We postulate that the responsible mutation lies in a gene that is upstream of MxaB in the lanthanide-regulatory cascade. In the Δ*xoxFS* mutant, we demonstrated that *mxaB* expression in the presence of lanthanum was similar to that of wild-type cells grown in the absence of lanthanum (Fig. 4C). Therefore, the regulatory cascade was broken upstream of *mxaB* expression in the Δ*xoxFS* mutant. We propose the existence of a lanthanum-binding regulatory protein that likely activates MxaB and other factors in the absence of lanthanum, and we propose that MxaB in turn activates the *mxa* operon and represses *xoxF* transcription.

In conclusion, we demonstrate that *M. buryatense* 5GB1C has a lanthanide-mediated switch regulating the choice between the two MDH enzymes, MxaFI and XoxF. This switch is mediated in part by the response regulator MxaB. Finally, the gene expression and MDH mutant growth rate analyses presented in this work lead us to postulate that XoxF is the major operational MDH in this type I methanotroph.

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