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XoxF Is Required for Expression of Methanol Dehydrogenase in *Methylobacterium extorquens* AM1▽

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In Gram-negative methylotrophic bacteria, the first step in methylotrophic growth is the oxidation of methanol to formaldehyde in the periplasm by methanol dehydrogenase. In most organisms studied to date, this enzyme consists of the MxaF and MxaI proteins, which make up the large and small subunits of this heterotetrameric enzyme. The *Methylobacterium extorquens* AM1 genome contains two homologs of MxaF, XoxF1 and XoxF2, which are ~50% identical to MxaF and ~90% identical to each other. It was previously reported that *xoxF* is not required for methanol growth in *M. extorquens* AM1, but here we show that when both *xoxF* homologs are absent, strains are unable to grow in methanol medium and lack methanol dehydrogenase activity. We demonstrate that these defects result from the loss of gene expression from the *mxa* promoter and suggest that XoxF is part of a complex regulatory cascade involving the 2-component systems MxcQE and MxbDM, which are required for the expression of the methanol dehydrogenase genes.

Methylobacterium extorquens AM1 is a methylotrophic bacterium ubiquitous in the environment and in particular on the undersides of leaves. This organism is able to metabolize both single-carbon compounds like methanol and multicarbon compounds like succinate and pyruvate (1, 11, 19). *M. extorquens* AM1 is a model organism for understanding the process of methylotrophic growth and has been studied biochemically and genetically for over 50 years (reviewed in reference 7). To utilize methanol as a sole source of carbon and energy, methanol is first oxidized to formaldehyde in the periplasm via methanol dehydrogenase, a soluble quinoprotein (2–4). This enzyme uses pyrroloquinoline quinone (PQQ) to sequentially transfer two electrons to cytochrome c_L, which enters the electron transport chain, resulting in ~1 molecule of ATP per molecule of methanol oxidized (9). Methanol dehydrogenase is a heterotetrameric protein consisting of two 66-kDa large subunits (MxaF) and two small 8.5-kDa subunits (MxaI) (28, 37). The large subunit contains the active-site residues and the PQQ prosthetic group, which is coordinated to a calcium ion in the active site (37). The loss of this enzyme in *M. extorquens* AM1 eliminates virtually all methanol dehydrogenase activity under any condition tested to date (27).

Previously, it was discovered that the *M. extorquens* AM1 genome contains a homolog of the large subunit, MxaF, which was named XoxF (6) based on its similarity to the XoxF protein of *Paracoccus denitrificans* (13). This protein is predicted to be a PQQ-dependent periplasmic alcohol dehydrogenase and is ~50% identical to MxaF. The region downstream of *xoxF* contains *xoxG*, which encodes a putative *c*-type cytochrome, and *xoxJ*, which has similarity to *mxaJ*, a gene of unknown function that was proposed previously to be required for the activation of methanol dehydrogenase and has similarity to periplasmic binding proteins (23, 35). Recently, it was shown in *M. extorquens* AM1 that mutations in *xoxF* lead to a 30% decrease in the growth rate in methanol medium and reduced methanol uptake and result in a fitness defect during plant colonization (31). XoxF was purified and shown to bind methanol with a high affinity (11 μM), yet the rate of methanol oxidation was ~10-fold lower than that with purified methanol dehydrogenase (31). The overexpression of this enzyme did not allow the growth of an *mxaF* mutant; thus, the *in vivo* function of XoxF in *M. extorquens* remained unclear (31).

Some organisms capable of methanol oxidation do not contain the MxaFI-type methanol dehydrogenase but do contain XoxF (10, 15, 18). In *Rhodobacter sphaeroides*, which does not contain an MxaFI-type methanol dehydrogenase, the loss of *xoxF* results in the loss of methanol-dependent oxygen uptake and the inability to use methanol as a photosynthetic carbon source, suggesting that XoxF may function as a methanol dehydrogenase in some organisms *in vivo* (38). In *Paracoccus denitrificans*, which contains the MxaFI-type methanol dehydrogenase, the loss of *xoxF* results in a partial growth defect in methanol medium (13). Protein expression studies have shown that in *Methylophaga* sp., which contains both MxaFI and XoxF, XoxF was highly abundant during growth on dimethylsulfide but not on methanol (30). However, XoxF is also found in nonmethylotrophic organisms, further complicating a predicted role for this enzyme in methylotrophic growth (10). As of yet, in *M. extorquens* AM1, no *in vivo* methanol dehydrogenase activity has been directly attributable to XoxF.

Recently, for a variety of other *Methylobacterium* species, Hibi et al. showed that the rare earth element (REE) La^{3+} increased methanol dehydrogenase activity up to 5.7-fold in cell extracts (14). Methanol dehydrogenase was purified from cells grown in media containing exogenous La^{3+} or Ca^{2+}, and upon sequencing, it was determined that the methanol dehydrogenase purified from La^{3+}-grown cells corresponded to XoxF, while the methanol dehydrogenase purified from Ca^{2+}-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
Strains		
AM1	Rif^r derivative (wild type)	27
AA31	*mxcE* (*moxE*[a]); chemically induced mutation	27
CM194.1	Δ*mxaF*	22
ES890	*xoxF1*::Km	This study, 6[b]
ES970	Δ*xoxF2*	This study
ES1022	Δ*xoxF2 xoxF1*::Km	This study
ES1100	Δ*mxaF* Δ*xoxF2 xoxF1*::Km	This study
ES1126	Δ*xoxF2 xoxF1*::Km$_{sup}$; spontaneously isolated suppressor mutant	This study
ES1258	*mxbM*::Km	This study
ES1291	*xoxG*::Km	This study
ES1296	Δ*xoxF2 xoxG*::Km	This study
Plasmids		
pAP3	Expression vector using the META1_3512 promoter (Tc^r)	This study
pAP5	Promoterless *yfp* fusion vector created from pCM62 (Tc^r)	This study
pALS24	pHX200 with *mxbDM* promoter region (Tc^r)	34
pAYC61	Allelic exchange suicide vector (Tc^r Ap^r)	5
pCM62	Expression vector using the *lac* promoter (Tc^r)	20
pCM80	Expression vector using the *lac* and *mxa* promoters (Tc^r)	20
pCM86	*mxaF* in pCM80 (Tc^r)	20
pCM157	*cre* expression vector (Tc^r)	21
pCM184	Allelic exchange suicide vector (Km^r Tc^r Ap^r)	21
pES134-135	pCM184 with *xoxF2* upstream and downstream flanks (Km^r Tc^r Ap^r)	This study
pES222-223	pCM184 with upstream and downstream *xoxG* flanks (Km^r Tc^r Ap^r)	This study
pES501	pHX200 with *mxcQE* promoter region (Tc^r)	This study
pES500	*xoxF1*, *xoxG*, and *xoxJ* cloned into pAP3 (Tc^r)	This study
pES502	pAP5 with *mxa* promoter region (Tc^r)	This study
pES503	pAP5 with *xox1* promoter region (Tc^r)	This study
pHX200	Promoterless *xylE* fusion vector (Tc^r)	39
pLC6168	pAYC61 with *xoxF1*upstream and downstream flanks (Km^r Tc^r Ap^r)	6

[a] The *mxcE* mutant strain was originally reported under the name *moxE*.
[b] The plasmid used to construct the *xoxF1*::Km mutant was constructed as described previously (6) and conjugated into the wild-type *M. extorquens* AM1 strain background used in this study.

grown cells corresponded to MxaFI (14). It has not yet been shown whether the effect of La^{3+} is indirect, possibly by the induction of a stress response system or an alteration of gene expression, or direct, by interacting with XoxF and participating in methanol oxidation.

Recently, the genome sequence for *M. extorquens* AM1 was reported (36), revealing a second XoxF paralog (XoxF2), which is also ~50% identical to MxaF and ~90% identical to XoxF, herein referred to as XoxF1. We show that the loss of both *xoxF1* and *xoxF2* leads to an inability to grow using methanol as a carbon and energy source and a nearly complete loss of methanol dehydrogenase activity, identical to the phenotype of an *mxaF* mutant strain. Using promoter fusion constructs, we demonstrate that expression of the genes encoding methanol dehydrogenase is severely repressed in the *xoxF1 xoxF2* double mutant strain and that this decrease in expression is likely all or in part due to the decreased expression of the 2-component systems *mxbDM* and *mxcQE*, which are required to activate the expression of the methanol dehydrogenase genes (34).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. extorquens* AM1 strains and plasmids used in this study are described in Table 1. *M. extorquens* AM1 strains were grown in a minimal salts medium (29) at 28°C to 30°C with methanol (0.5%) and/or succinate (0.4%) as a carbon source. Difco nutrient broth supplemented with Difco BiTek agar (1.5%) was used for conjugation between *Escherichia coli* helper strain S17-1 (32) and *M. extorquens* AM1. *E. coli* strains were grown at 37°C on Luria-Bertani agar. When appropriate, antibiotics were added to the following concentrations: tetracycline (Tc) at 10 μg/ml, kanamycin (Km) at 50 μg/ml, and rifamycin (Rif) at 50 μg/ml.

Strain construction. Null mutations were generated in *xoxF2* and *xoxG* using the allelic exchange suicide vector pCM184 as described previously (21). A deletion mutant in *xoxF2* was made by the introduction of the *cre* expression vector pCM157 into the *xoxF2*::Km mutant strain (21), followed by plasmid curing. Null mutations in *xoxF1* and *mxbM* were made by using pAYC61 as described previously (5), and mutations were confirmed by diagnostic PCR. *xoxF1 xoxF2* suppressor mutant strains were spontaneously isolated from all 3 flasks during the growth curve analysis shown in Fig. 1A and described below. After the isolation of the *xoxF1 xoxF2* suppressor mutants, the Km insertion in *xoxF1* and the absence of *xoxF2* were confirmed by diagnostic PCR.

Phenotypic analysis. (i) Growth. Growth was assessed for 3 biological replicates both by scoring the colony size on agar plates and by growth curve analysis monitoring the optical density (OD) at 600 nm using a Shimadzu (Kyoto, Japan) UV-2401 PC spectrophotometer. For growth curve analysis, strains were grown at 30°C to late log phase, subcultured (2 ml) into 100 ml of minimal medium in 250-ml flasks containing the appropriate carbon source, and shaken at 200 rpm. For carbon source transition experiments, strains were grown in 100 ml of minimal succinate medium to an OD of ~0.6 using the conditions described above, pelleted, washed in minimal medium, and resuspended in 100 ml minimal medium containing methanol.

(ii) Complementation studies. To create a high-level-expression vector, the promoter region of RMQ02531/META1_3512, which was shown in previous microarray experiments to be highly expressed and constitutive in all available array data (29, 33; E. Skovran, unpublished data), was amplified and cloned into the AclI site of pCM62, creating pAP3. The *xox1* operon was amplified and inserted into the EcoRI and KpnI sites of pAP3, creating pES500. Complementation was assessed in liquid and on solid medium containing 0.5% methanol.

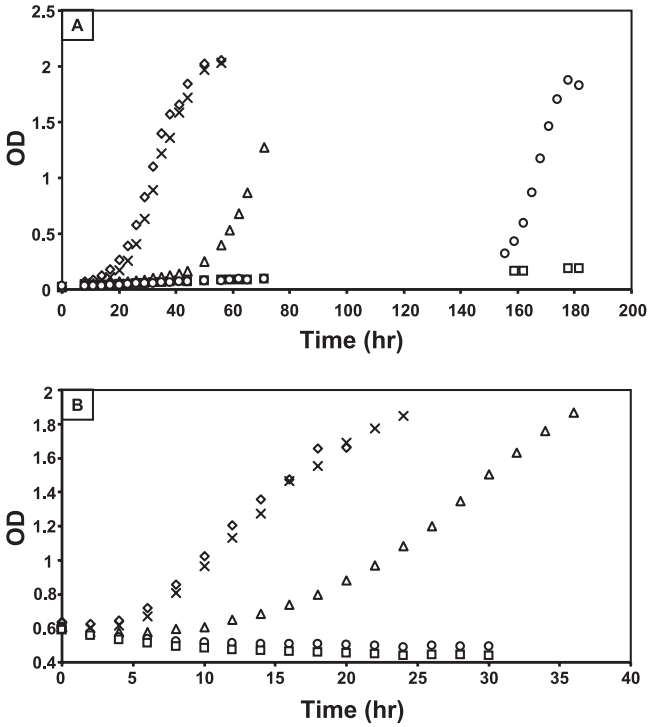

FIG. 1. Phenotypic growth defects in the *xoxF* and *mxaF* mutant strains. (A) Growth of wild-type (diamonds), *mxaF* (squares), *xoxF1* (triangles), *xoxF2* (X's), and *xoxF1 xoxF2* (circles) strains in minimal medium containing 120 mM methanol as a sole source of carbon. Growth seen past 140 h for the *xoxF1 xoxF2* double mutant strain (circles) was due to a second site suppressor mutation. (B) Growth of wild-type (diamonds), *mxaF* (squares), *xoxF1* (triangles), *xoxF2* (X's), and *xoxF1 xoxF2* (circles) strains after transition of the cells from succinate growth at an OD of 0.6 to growth on methanol. Graphs depict representative data from three biological replicates. The rate of growth varied by ≤8% between replicates.

Enzyme assays. Cells and cell extracts for enzyme assays were prepared by using a French press as previously described (33). A minimum of 3 biological replicates was used to assay methanol dehydrogenase and catechol dioxygenase (XylE). Assays were performed as previously described (for methanol dehydrogenase [12] and XylE [34]), except that methanol dehydrogenase and XylE were measured in a final volume of 200 μl in a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA) at room temperature. Absorbance data were converted to a 1-cm path length by dividing the slope by the conversion factor, 0.541. Protein concentrations were determined by using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). The visualization of methanol dehydrogenase activity was achieved by the isoelectric focusing of cell extracts using a pH range of 3 to 9 and incubation with nitroblue tetrazolium and phenazine methosulfate as described previously (6), except that 20 mM methanol and 15 mM ammonium chloride were used as substrates.

Protein separation. Cell extracts were prepared as described above and separated (5 to 15 μg) by using standard SDS-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel and staining with Coomassie blue R. Precision Plus Protein Kaleidoscope prestained standards (Bio-Rad, Hercules, CA) were used for size estimations.

Promoter fusion assays. (i) Yellow fluorescent protein promoter fusions. A modified version of yellow fluorescent protein (YFP) that is brighter and more stable in response to pH changes was amplified from the Venus construct (25) by PCR and inserted into pCM62 (20) in the opposite orientation of the *lac* promoter using the SacI restriction site to generate pAP5. The promoter regions for the *mxa* operon and the *xox1* operon were cloned into the AclI site, creating plasmids pES502 and pES503, respectively. Strains containing the *yfp* promoter fusion constructs were grown at 29°C using a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA) with continuous shaking in a Nunc black optical 96-well microtiter plate with a clear bottom (Nalge Nunc International, Rochester, NY). Cells were grown to the mid-log phase, at which point the relative fluorescence units (RFU) were measured by using an Infinite F500 instrument (Tecan, Männedorf, Switzerland) with excitation and emission spectra of 485 and 535 nm, respectively. The RFU/OD is reported.

(ii) *xylE* promoter fusions. The promoter region for the *mxcQE* genes was cloned into the KpnI and SacI sites in pHX200 (39), creating pES501. Catechol dioxygenase activity was measured as described above.

RESULTS

Loss of both *xoxF1* and *xoxF2* results in growth phenotypes identical to that of a methanol dehydrogenase null mutant strain (*mxaF*). Methanol dehydrogenase is required for the oxidation of methanol, resulting in the inability of an *mxaF* null mutant strain to grow on methanol medium, but the ability of *mxaF* null mutant strains to grow using other C_1 carbon sources is not altered. To estimate the growth phenotypes of the *xox* mutant strains, single colonies of the mutant strains were compared for colony size to the wild-type and the *mxaF* mutant strains on minimal medium containing methanol and other C_1 carbon sources (methylamine and formate) (Table 2). The *xoxF1* single mutant resulted in a partial growth defect on methanol medium, whereas the *xoxF2* single mutant showed no observable growth defect. However, the *xoxF1 xoxF2* double mutant strain displayed growth phenotypes identical to that of the *mxaF* mutant strain: an inability to grow on methanol medium but wild-type growth with the other C_1 carbon sources methylamine and formate.

The *xoxF1* gene is in a putative operon with a cytochrome, *xoxG*, and a putative periplasmic binding protein, *xoxJ*. To test if the cytochrome was also required for methanol growth in addition to the XoxF subunit, strains lacking *xoxG* in both the wild-type and *xoxF2* backgrounds were constructed. Interestingly, growth of the *xoxG* single and *xoxF2 xoxG* double null mutant strains was similar to that of the wild type, suggesting that only the homolog of the methanol dehydrogenase large subunit is required for methanol growth (Table 2).

Since purified XoxF has methanol dehydrogenase activity *in vitro* (10-fold decrease in the V_{max} compared to that of methanol dehydrogenase [31]), it is possible that XoxF is a methanol dehydrogenase functioning *in vivo* under unknown conditions. To test whether XoxF and MxaF could substitute for one

TABLE 2. Growth and complementation of the *xox* and *mxaF* mutant strains[a]

Strain	Growth with carbon source			Complementation in methanol medium			
	MeOH	MA	Formate	pCM80[b]	pCM86	pAP3[c]	pES500
WT	++	++	+	++	++	++	++
mxaF	−	++	+	−	+	−	−
xoxF1	+	++	+	ND	ND	ND	ND
xoxF2	++	++	+	ND	ND	ND	ND
xoxF1 xoxF2	−	++	+	−	−	−	++
xoxG	++	++	+	ND	ND	ND	ND
xoxF2 xoxG	++	++	+	ND	ND	ND	ND

[a] Abbreviations are as follows: MeOH, methanol; MA, methylamine; WT, wild type; ND, not determined. −, no growth; + or ++, growth (a single plus indicates that colony size is about half that seen for a wild-type strain on methanol medium).

[b] pCM80 is the empty parent vector for pCM86, which harbors *mxaF* (20).

[c] pAP3 is the empty parent vector for pES500, which harbors the *xoxF*, *xoxG*, and *xoxJ* genes.

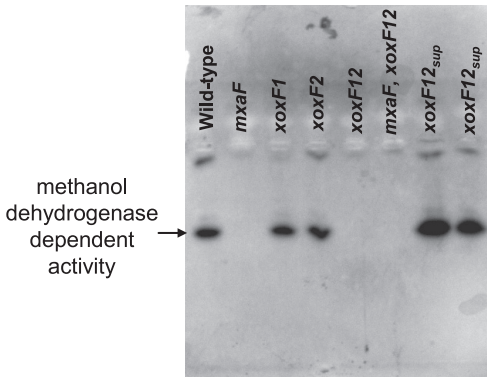

FIG. 2. Visualization of methanol dehydrogenase activity in the *xoxF* and *mxaF* mutant strains using isoelectric focusing gel electrophoresis. Cells were grown in minimal medium containing both succinate and methanol, and cell extracts from these strains were isoelectrofocused using a pH range of 3 to 9. The presence of methanol dehydrogenase activity was detected by using nitroblue tetrazolium and phenazine methosulfate in the presence of 20 mM methanol and 15 mM ammonium chloride. $xoxF12_{sup}$ refers to two different *xoxF1 xoxF2* suppressor mutants that were spontaneously isolated.

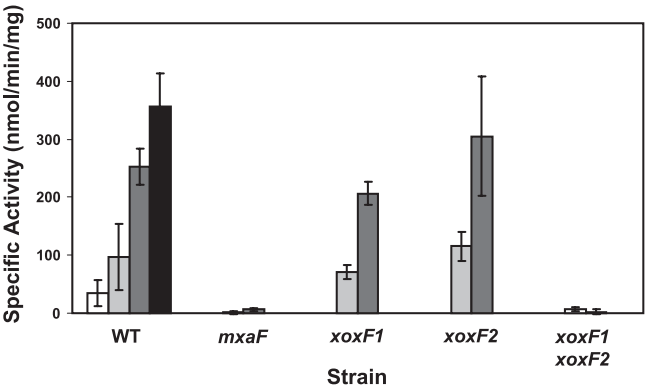

FIG. 3. Enzyme activities in the *xoxF* and *mxaF* mutant strains. Methanol dehydrogenase activities were measured in cell extracts 4 h (light gray) and 10 h (dark gray) after the transition from succinate to methanol growth. Activities in succinate-grown cells (white bars) and methanol-grown cells (black bars) are shown for the wild-type strain as a reference.

another, pCM86 containing *mxaF* and pES500 containing the *xox1* cluster (*xoxF1*, *xoxG*, and *xoxJ*) were conjugated into the *mxaF* and *xoxF1 xoxF2* mutant strains and tested for growth on solid methanol medium (Table 2) and liquid methanol medium (data not shown). Under both conditions, pCM86 containing *mxaF* allowed the partial growth of the *mxaF* mutant strain, as previously described (20), but did not complement the *xoxF1 xoxF2* double mutant strain. pES500 restored wild-type growth to the *xoxF1 xoxF2* mutant strain but did not allow the growth of the *mxaF* mutant strain, confirming that these two similar dehydrogenases cannot substitute for each other under the conditions tested, even when expressed from a complementing plasmid. These results demonstrate that both *mxaF* and *xox* have unique and separate roles in methanol metabolism.

For a more detailed analysis of the growth defect in methanol medium, *xoxF* single and double mutant strains were tested for growth in liquid methanol minimal medium (Fig. 1A) and for their growth responses when the cells were transitioned from succinate to methanol growth during the mid-log phase (Fig. 1B). The *xoxF2* mutant strain displayed no phenotypic defect, while the *xoxF1* mutant had a slight defect in the growth rate and a reproducibly increased lag (~50 h) compared to those of the wild-type strain. Like the *mxaF* mutant, the *xoxF1 xoxF2* double mutant strain could not grow in methanol medium. However, suppressor mutations consistently arose ~150 h postinoculation in the *xoxF1 xoxF2* double mutant but not the *mxaF* mutant strain. Upon retesting, these spontaneous mutants grew like the wild-type strain in methanol medium (data not shown) and are subsequently referred to as *xoxF1 xoxF2*$_{sup}$ in the text below. Because these suppressor mutants were easily obtained, it seemed likely that the suppression was due to a loss of the function of an unknown gene. Attempts to identify this gene using transposon mutagenesis were unsuccessful. Suppressor mutants bearing a transposon were isolated, but sequencing from the transposons resulted in multiple different sequence identifications and were seemingly random. When *M. extorquens* AM1 is under stress, many of the ~200 insertion elements contained in the *M. extorquens* genome are induced (24), thus making it difficult to use transposon mutagenesis as a method for suppressor identification.

In a previous study, when wild-type chemostat-grown cells were transitioned from succinate growth to methanol growth, the expression levels of both *xoxF* genes increased, while the expression levels of the *mxa* genes decreased (33). To determine how the growth of the *xoxF* mutant strains would respond when the carbon source was switched from succinate to methanol, cells were grown with succinate to an OD of 0.6, washed, and resuspended in methanol medium. The growth of the *xoxF1* mutant after the transition showed a larger defect (Fig. 1B) than that in methanol-grown cells alone (Fig. 1A), and the growth of the *xoxF1 xoxF2* double mutant strain was identical to that of the *mxaF* deletion strain, with a continual slight decrease in OD after the transition (Fig. 1B). Taken together, these growth phenotypes are consistent with XoxF acting at the methanol oxidation step.

XoxF is required for methanol dehydrogenase activity in cell extracts. To determine if the cause of the growth defect in the *xoxF1 xoxF2* double mutant strain was due to a lack of methanol dehydrogenase activity, cells were grown in medium containing succinate and methanol, and the methanol dehydrogenase activity in cell extracts was visualized by using native isoelectric focusing gel electrophoresis and staining with nitroblue tetrazolium and phenazine methosulfate in the presence of methanol and ammonium chloride (Fig. 2). Methanol dehydrogenase-dependent activity was absent in both the *mxaF* and *xoxF1 xoxF2* double mutant strains but was restored in the *xoxF1 xoxF2* suppressor mutant isolates. The absence of a single identical band in both the *mxaF* and *xoxF1 xoxF2* mutant strains is suggestive that both XoxF and MxaF are required for the activity of the same methanol dehydrogenase enzyme.

To measure the amount of methanol dehydrogenase activity in the *xoxF* mutant strains, cells were grown in succinate medium to the mid-log phase (OD of 0.6), washed and resuspended in methanol medium, and harvested at 4 and 10 h posttransition for activity measurements (Fig. 3). Activities in succinate- and methanol-grown cells were also measured for

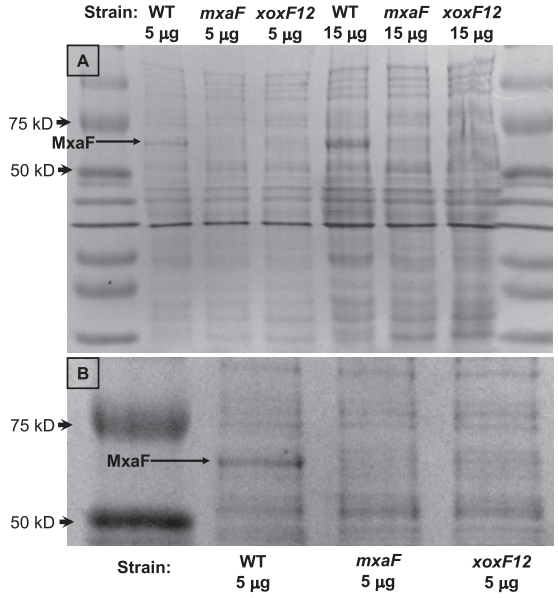

FIG. 4. Coomassie-stained 12.5% SDS-polyacrylamide gel containing cell extracts from *M. extorquens* AM1 strains. The size of the MxaF subunit of methanol dehydrogenase is 66 kDa. (A) Entire gel shown containing 5 and 15 μg of cell extract. (B) Enlarged portion of the gel shown in panel A.

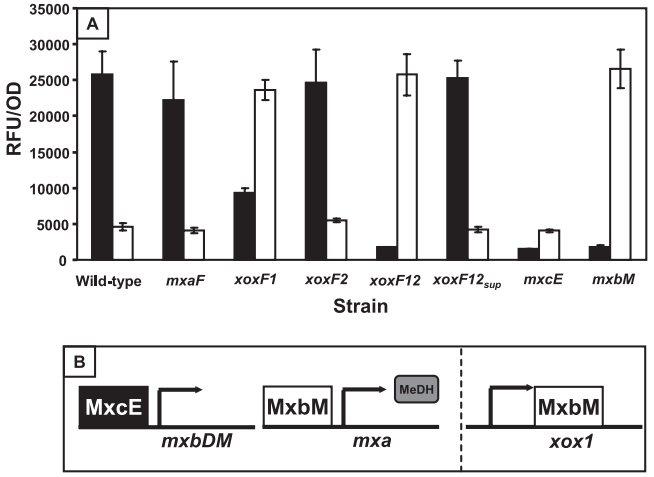

FIG. 5. Expression of *mxaF-yfp* and *xox1-yfp* promoter fusions in *M. extorquens* AM1 strains. (A) *mxa* (black) and *xox1* (white) promoter activities were determined by calculating the relative fluorescence units (RFU) per OD from 3 biological replicates grown to the mid-log phase (OD of ~0.6) in medium containing both succinate and methanol. The background level from a promoterless *yfp* fusion vector was 3,940 ± 70 RFU/OD. (B) Current model for how MxcE and MxbM regulate expression of the *mxa* operon. In this model, both the MxcE and MxbM response regulators are required for the expression of the *mxa* operon, with MxcE having an indirect effect. MeDH represents production of the methanol dehydrogenase gene. MxcE has been shown to be involved in the activation of expression from the *mxbDM* promoter region, predicting a cascade of regulation, with MxcE activating the expression of *mxbM*, which, when translated, activates expression from the *mxa* promoter. The data in panel A added to the model shown passed the dashed line, suggesting that MxbM may act as a repressor of the *xox1* operon. The direct binding of both MxcE and MxbM has not yet been demonstrated.

the wild-type strain. The level of methanol dehydrogenase activity was low in wild-type succinate-grown cells and increased over time, similar to the results for chemostat-grown cells transitioned from succinate to methanol growth (33) (Fig. 3A). As was previously reported (27), strains lacking *mxaF* showed only trace amounts of methanol dehydrogenase activity. Strains lacking both *xoxF1* and *xoxF2* also had only trace amounts of methanol dehydrogenase activity, suggesting that both *mxa* and *xox* are required for the same methanol dehydrogenase step (Fig. 3A).

The loss of either *xoxF1* or *xoxF2* by itself resulted in no major defect in methanol dehydrogenase activity during the transition (Fig. 3A), suggesting that XoxF1 and XoxF2 can substitute for one another, and it is only when both are lacking that this defect is severe enough to be observed during the transition.

Levels of the MxaF protein are decreased in the *xoxF1 xoxF2* double mutant strain. It seemed unlikely that the requirement for Xox was to function directly as a methanol dehydrogenase under the conditions tested, since the loss of the MxaFI methanol dehydrogenase eliminated both growth and activity. This result indicated that XoxF might be required for the activation of methanol dehydrogenase or for the production of the methanol dehydrogenase protein. To determine if methanol dehydrogenase was produced (but not active) in the *xoxF1 xoxF2* double mutant strain, proteins in cell extracts were separated by using SDS-polyacrylamide gel electrophoresis. Since MxaF is one of the most abundant proteins in the cell, it is visible after staining with Coomassie blue (Fig. 4). The loss of both *xoxF1* and *xoxF2* greatly reduced the band corresponding to MxaF, suggesting that XoxF is required for the expression or stability of the methanol dehydrogenase enzyme.

Expression from the *mxa* and *xox1* promoter regions. To examine the expressions of the *mxa* and *xox* operons, transcriptional promoter fusions were created by the insertion of the *mxa* and *xox1* promoter regions upstream of a stable promoterless version of *yfp*. The RFU/OD was measured for the *xoxF* and *mxaF* mutant strains and for strains lacking the response regulators shown to be required for the expression of the *mxa* operon (34) (Fig. 5A). A model of our current understanding of the regulatory cascade required for *mxa* expression is depicted in Fig. 5B. In minimal medium containing both succinate and methanol, the wild-type strain exhibited high levels of expression from the *mxa* promoter (25,800 RFU/OD; average of triplicates) and just above background expression levels from the *xox1* promoter region (4,600 RFU/OD; average of triplicates) (Fig. 5A). Background expression was defined as the RFU/OD detected from the promoterless *yfp* fusion construct pAP5 (3,940 RFU/OD; average of triplicates). A promoter fusion construct was also generated by using a large region upstream and partially within the *xoxF2* gene; however, YFP fluorescence measured under multiple growth conditions and strain backgrounds showed only background levels. Thus, it was undetermined if the level of expression of *xoxF2* was extremely low in all cases or if the promoter region was located elsewhere. Wild-type levels of expression were observed from both promoters in the *mxaF* and *xoxF2* mutant strains. However, the level of expression from the *mxaF* promoter was

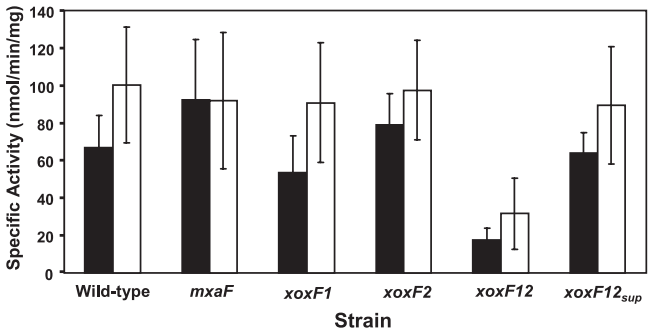

FIG. 6. Expression of *mxc-xylE* and *mxb-xylE* promoter fusions in *M. extorquens* AM1 strains. *mxc* (black) and *mxb* (white) promoter activities were determined by measuring catechol 2,3-dioxygenase activity (XylE) in crude cell extracts of strains grown in medium containing both succinate and methanol. Cell extracts containing the promoterless *xylE* construct pHX200 had a background activity of 10.9 ± 2.6 nmol min^{-1} mg $protein^{-1}$.

decreased in the *xoxF1* mutant strain and reduced to below background levels in the *xoxF1 xoxF2* double mutant strain, confirming that XoxF is required for the expression of methanol dehydrogenase. Of interest, the loss of *xoxF1* resulted in increased expression from the *xox1* promoter to levels observed for the *mxa* promoter. The *xoxF1 xoxF2* suppressor mutants that were spontaneously isolated and described above had wild-type levels of expression from both promoters, explaining their ability to grow in methanol medium.

As controls, the activity from these promoters was also measured in the response regulator mutants that were previously shown to be required for the expression of the *mxa* genes (34) (Fig. 5A). As expected, expression from the *mxa* promoter was severely repressed, resulting in below-background levels of fluorescence detected in each of the response regulator mutant strains (*mxcE* and *mxbM*). Expression from the *xox1* promoter was unaffected in the *mxcE* mutant strain but, interestingly, was derepressed in the *mxbM* mutant to the high levels seen for the *mxa* promoter in the wild type and for the *xox1* promoter in the *xoxF1* mutant strains, suggesting that *mxa* and *xox1* are oppositely regulated by the MxbDM 2-component regulatory system. This opposite expression of the *mxa* and *xox* genes was also observed previously during the transition from succinate to methanol growth (33).

Expression of the 2-component regulatory systems required for activation of the *mxa* genes is repressed in the *xoxF1 xoxF2* double mutant strain. Because *xoxF* is required for the expression of the *mxa* genes, it is possible that the XoxF proteins are exerting their effects on regulation through one or more of the 2-component regulatory systems required for the activation of the *mxa* genes. To test if the expression of the 2-component systems is altered in the *xoxF* mutant strains, the promoter regions of *mxcQE* (this work) and *mxbDM* (34) were cloned upstream of *xylE* in pHX200 (39) and conjugated into the various *mxaF* and *xoxF* strain backgrounds. Only the *xoxF1 xoxF2* double mutant strain showed significantly altered expression (Fig. 6). The level of expression from the *mxc* promoter was decreased to slightly above background levels (17.5 nmol min^{-1} mg $protein^{-1}$, versus a background level of 10.9 nmol min^{-1} mg $protein^{-1}$, measured using the promoterless *xylE* construct pHX200), while the level of expression from the *mxb* promoter was decreased to 31.6 nmol min^{-1} mg $protein^{-1}$. Expression levels from both promoters increased to wild-type levels in the *xoxF1 xoxF2* suppressor strains.

DISCUSSION

The presence of *xoxF* is widespread in methylotrophs, and it exists even in some nonmethylotrophic bacteria. It is only recently that some understanding of the role of these methanol dehydrogenase-like enzymes has begun to emerge. In organisms like *Rhodobacter sphaeroides*, in which no MxaFI-type methanol dehydrogenase exists, XoxF was implicated as a methanol dehydrogenase, but methanol dehydrogenase activity in that bacterium was not detected *in vivo*, thus leaving open the question of whether XoxF was directly or indirectly required for growth with methanol. Schmidt et al. demonstrated that *in vitro*, XoxF from *M. extorquens* did indeed have methanol dehydrogenase activity, although the rate of oxidation was low (31). Thus, it was unclear why XoxF was unable to substitute for the methanol dehydrogenase *in vivo* when expressed from a multicopy plasmid.

In *M. extorquens* AM1, the existence of *xoxF1* has been known for ~15 years (6), but until the genome was sequenced (36), the presence of the second homolog, *xoxF2*, was undiscovered. Here we showed that it is only when both *xoxF1* and *xoxF2* are absent that a strict requirement for growth in methanol medium is uncovered and that this requirement can be explained by a role for XoxF in the expression of the *mxa* operon encoding methanol dehydrogenase. The presence of the *mxaG* cytochrome homolog (*xoxG*) downstream of *xoxF1* suggests that XoxF may function as an active dehydrogenase, yet the loss of *xoxG* had no detectable effect on the ability of *M. extorquens* AM1 to grow in methanol medium. This suggests either that the dehydrogenase activity is not required for the regulatory role of XoxF or that another cytochrome like MxaG may be able to substitute for XoxG. It is possible that XoxF has a dual function, acting both in a catalytic and in a regulatory role.

One possibility for the role of XoxF is to act as an environmental sensor, possibly by binding to or producing a signal molecule and exerting its effect through a 2-component system. That signal molecule must not be methanol, because a significant expression of the *mxa* operon occurs in the absence of methanol (33). Free formaldehyde is also unlikely to be the signal produced by XoxF, since the addition of formaldehyde does not allow the growth of the *xoxF1 xoxF2* double mutant strain in methanol (data not shown). However, this does not exclude the ability of formaldehyde bound to XoxF to function as the signal that begins the regulatory cascade. However, this is unlikely since there is a high level of expression of methanol dehydrogenase in succinate-grown cells, where formaldehyde should not be present in the periplasm at significant levels (33).

If XoxF does act as an environmental sensor, the binding of a signal molecule may be what begins the regulatory cascade involving the expression of the 2-component systems MxbDM and MxcQE, required for the expression of methanol dehydrogenase. A complex feedback loop appears to be built into this regulatory cascade since (i) Xox is required for normal expression levels of both *mxcQE* and *mxbDM*, (ii) MxbDM decreases

the expression levels of *xox1* (either directly or indirectly), and (iii) MxcQE is required for the expression of *mxbDM* (34), creating a complex and interwoven regulation scheme. Such a mode of regulation might be very sensitive to changes in the environment, allowing a quick response when needed, or provide a robustness to the system, maintaining homeostasis when reached. The isolation of *xoxF1 xoxF2* suppressor mutants suggests that there are other components involved in the regulation of methanol dehydrogenase that are yet unknown. In these suppressor mutants, expression levels of *mxa* and *xox1* as well as expression from the *mxbDM* and *mxcQE* promoters were returned to wild-type levels (Fig. 5 and 6), suggesting a loss of a repressor. Further work is needed to determine the specific interactions between the known regulatory components and to identify additional unknown regulatory components involved in the regulation of expression of the methanol dehydrogenase genes.

We demonstrate an absolute requirement for XoxF in the utilization of methanol, specifically at the methanol dehydrogenase step. This requirement is due to a role for XoxF in the expression of the methanol dehydrogenase genes. An examination of expression from the promoters of the known regulators of methanol dehydrogenase demonstrates that XoxF is part of a complex regulatory cascade. This does not exclude XoxF from having a second function, possibly in the oxidation of methanol under unknown conditions or in the oxidation of another unknown substrate.

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