Molecular analysis of \textit{mxbD} and \textit{mxbM}, a putative sensor–regulator pair required for oxidation of methanol in \textit{Methylobacterium extorquens} AM1

Amy L. Springer,¹ Christina J. Morris¹† and Mary E. Lidstrom¹⁺

Author for correspondence: Mary E. Lidstrom. Tel: +1 206 616 5282. Fax: +1 206 616 5721.
e-mail: lidstrom@cheme.washington.edu

Five genes are thought to be required for transcription of methanol oxidation genes in \textit{Methylobacterium} strains. These putative regulatory genes include \textit{mxcQE}, which encode a putative sensor–regulator pair, and \textit{mxbDM} and \textit{mxaB}, whose functions are less well-understood. In this study, \textit{mxbDM} in \textit{Methylobacterium extorquens} AM1 were shown to be required for expression of a \textit{xyIIE} transcriptional fusion to the structural gene for the large subunit of methanol dehydrogenase (\textit{mxaF}), confirming the role of these genes in transcriptional regulation of \textit{mxaF}. The nucleotide sequence suggests that \textit{mbD} encodes a histidine protein kinase with two transmembrane domains and that \textit{mxbM} encodes a DNA-binding response regulator. A \textit{xyIIE} transcriptional fusion to the putative \textit{mbD} promoter showed low-level expression in wild-type cells grown on one-carbon (C₁) compounds and no detectable expression in cells grown on succinate. Deletion analysis of this promoter construct showed that the region 229–129 bp upstream of the start of \textit{mbD} is required for expression. The expression of the \textit{mbD–xyIIE} fusion was examined in each of the five known regulatory mutant classes. \textit{xyIIE} expression was reduced to non-detectable levels in \textit{MxcQ} and \textit{MxcE} mutants, but was not affected in the other regulatory mutants or in non-regulatory mutants defective in methanol oxidation. These results suggest a regulatory hierarchy in which the sensor–regulator pair \textit{MxcQE} control expression of the sensor–regulator pair \textit{MxbDM}, and \textit{MxbDM} in turn control expression of a number of genes involved in methanol oxidation.

Keywords: methylotroph, methanol oxidation, sensor kinase, response regulator

INTRODUCTION

\textit{Methylobacterium extorquens} AM1 is a pink-pigmented facultative methylotroph that can grow on methanol as its sole source of carbon and energy. Growth on methanol requires the quinoprotein methanol dehydrogenase (MDH), which oxidizes methanol to formaldehyde (Anthony, 1986). Formaldehyde can then be further oxidized for energy or assimilated into cell carbon (Anthony, 1986). MDH is an \(\alpha_2\beta_2\) tetramer that contains calcium at the active site and requires the prosthetic group pyrroloquinoline-quinone (PQQ) (Anthony, 1986; Xia \textit{et al.}, 1992; Richardson & Anthony, 1992; White \textit{et al.}, 1993; Anthony \textit{et al.}, 1994; Ghosh \textit{et al.}, 1995).

Genetic analyses of methanol oxidation in \textit{M. extorquens} AM1 and other methylotrophs have identified at least 26 genes required for the oxidation of methanol by MDH (Bastien \textit{et al.}, 1989; van Spanning \textit{et al.}, 1991; Morris \textit{et al.}, 1994, 1995; Springer \textit{et al.}, 1995). These methanol oxidation genes have been mapped to four different loci on the \textit{M. extorquens} AM1 chromosome: \textit{mxa}, \textit{mxb}, \textit{mxc} and \textit{mxd} (Lidstrom \textit{et al.}, 1994). The first of these loci contains a cluster of twelve known

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¹ Department of Chemical Engineering, Box 351750, and Department of Microbiology, Box 357242, University of Washington, Seattle, WA 98195-1750, USA

Abbreviations: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline-quinone.

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³ Present address: Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay Road, Kowloon, Hong Kong.

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genes: mxaF/GIRSACKLD (van Spanning et al., 1991; Morris et al., 1995), with an additional gene (mxaW) adjacent to mxaF and divergently transcribed (Xu et al., 1993; Lidstrom et al., 1994). The 66 and 9 kDa subunits of MDH are encoded by mxaF and mxaI, respectively, and mxaG encodes the associated cytochrome c$_5$ electron acceptor (Nunn & Lidstrom, 1986a, b; Anderson & Lidstrom, 1988; Nunn & Anthony, 1988). At least four of these genes, mxaACKL, are required for proper insertion of calcium into the active site (Richardson & Anthony, 1992; Morris et al., 1995), and mxaB is required for transcription of mxaF in both M. extorquens AM1 (Morris & Lidstrom, 1992) and Methylobacterium organophilum XX (Xu et al., 1993). The remaining genes in this cluster are of unknown function, but do not appear to be involved in regulation of methanol oxidation. The function of mxaW is unknown.

Two more loci contain two genes each, mxbDM and mxcQF, which are required for expression of mxaF in Methylobacterium strains (Springer et al., 1995; Xu et al., 1993). Another locus identified in M. extorquens AM1 contains at least two genes, mxdRS, which are of unknown function but are not involved in mxaF expression (Springer et al., 1995). In addition, there are two loci in M. extorquens AM1 containing at least seven genes, called pqq genes, required for biosynthesis of PQQ (Morris et al., 1994).

The transcription start site for mxaF has been mapped in both M. extorquens AM1 and M. organophilum XX, and expression has been studied in M. extorquens AM1 using an mxaF–lacZ fusion from a promoter probe vector (Morris & Lidstrom, 1992) and in M. organophilum XX using an mxaF–xylE fusion (Xu et al., 1993). The amounts of MDH protein of mxaF transcript, and of β-galactosidase activity from the mxaF–lacZ fusion are about ten-fold higher in cells grown on methanol than in cells grown on succinate in M. extorquens AM1, although the mechanism for this induction phenomenon is not known (Lauffer & Lidstrom, 1992; Morris & Lidstrom, 1992). Other C$_1$ compounds such as methylvamine will also induce MDH, even though MDH is not required for growth on these other substrates (Lauffer & Lidstrom, 1992). All three sets of regulatory genes are required for expression of the M. organophilum XX mxaF–xylE fusion in both M. organophilum XX and M. extorquens AM1 (Xu et al., 1993). These regulatory genes are interchangeable in these two Methylobacterium strains (Bastien et al., 1989; Springer et al., 1995) and presumably carry out similar functions. In addition, in M. extorquens AM1, MxbD, MxbM and MxaB have been shown to be required for normal expression of a pqqD–xylE fusion (Ramamoorthi & Lidstrom, 1995), and in M. organophilum XX, MxbD and MxbM are required for expression of an mxaW–xylE fusion (Xu et al., 1993). The mxb and mxc genes may also be involved in regulation of a novel c-type cytochrome, cytochrome c$_5_{537}$, which is present at elevated levels in Mxb and Mxc mutants of M. extorquens AM1 (Nunn & Lidstrom, 1986b; Day et al., 1990; Lee et al., 1991; Springer et al., 1995). This cytochrome has been purified, but its function is unknown (Anthony, 1992).

Two sensor kinase–response regulator pairs are known to be involved in mxaF transcription in methylo trophs. mxcQ and mxcE have been sequenced in M. organophilum XX and the encoded polypeptides share identity with NarX and NarP, regulators of anaerobic respiration in Escherichia coli (Xu et al., 1995). MxaY and MxaX regulate mxaF in Paracoccus denitrificans, also share identity with the NarX/NarP subfamily (Harms et al., 1993), and share identity with MxcQ and MxcE, respectively (Xu et al., 1995). It is not known if they are functionally interchangeable. Recent data have shown that while mxaX is required for transcription of mxaF, mxaY can be deleted with no effect on mxaF expression, suggesting that there may be an alternative protein kinase that phosphorylates MxaX in P. denitrificans (Yang et al., 1995). This is in contrast to MxcQ, which is required for transcription of mxaF in both Methylococcus strains.

Since MxbD and MxbM are involved in the expression of a number of methanol oxidation genes, we have studied the genes encoding these proteins in more detail. This paper reports the nucleotide sequence of the mxbDM region, indicating these genes encode a sensor–regulator pair different from MxcQF, and presents an analysis of the effects of the regulatory mutants on mxbD and mxaF promoters.

**METHODS**

**Bacterial strains and plasmids.** All methanol oxidation mutants were derived from rifamycin-resistant M. extorquens AM1 (Nunn & Lidstrom, 1986a). The following M. extorquens AM1 strains were used for transcriptional analyses: MxbM, 7-20; MxbD, 7-19; MxcQ, 8-10; MxcE, 7-12; MxdS, EM552. M. organophilum XX strains SM2 (MxbN mutant) and SM3 (MxbD mutant) were provided by R. S. Hanson (Machlin et al., 1987). The mxbD–xylE fusions are shown in Fig. 4. pALS18 was derived from the 0.5 kb BamHI–XmnI fragment (Fig. 2), which was cloned into pUC19 to make pALS122. The insert was then cut out with HindIII and BamHI and cloned into those sites in the promoter probe vector pHX200 (provided by R. S. Hanson and described by Xu et al., 1993). pALS22 and pALS23 were made by PCR amplification of the insert in pALS18 using the M13 forward primer designed for pUC19 (New England Biolabs), and each of two primers (AS-90 and AS-120, Fig. 5) that hybridize to regions upstream of mxbD. The amplified fragments were cloned using the TA cloning kit (Invitrogen). The inserts were then cut out with XbaI and BamHI and inserted into those sites of pHX200. pALS24 was derived from a 0.8 kb Spbl–BamHI fragment (Fig. 2) in pALS128. This insert was cut out with HindIII and BamHI and cloned into those sites in the promoter probe vector pHX200. The mxaF–xylE fusion pCM Bam12 was made using the 1.55 kb Xhol–SalI fragment from a 410XSpro7 (Morris & Lidstrom, 1992), which was cloned into the SalI site of pUC7 (Vieira & Messing, 1982), cut out with BamHI, and inserted into the BamHI site of pHX200 to make pCM Bam12 (Fig. 1). pALS12 was a derivative of pRK310 (Tc, IncP1; Ditta et al., 1985). The mobilizing plasmid used for conjugation was pRK2073 (Sm, Figurski & Helinski, 1979). E. coli strain DH5a (BRL) was used for DNA
Facility with an Applied Biosystems model 3730 automated Geneclean reagents (BiolOli). Enzymes were obtained from plus 0.2 g from these subclones using the M13 forward and reverse 50 mM Tris buffer solution (pH 8). Enzymes were obtained from Sigma. E. coli strains used for subcloning and sequence analyses were grown on L broth (Sambrook et al., 1989) containing 50 g ampicillin l-1. Nutrient agar and Bacto-agar were obtained from Difco.

**Media and growth conditions.** Methyllobacterium strains were grown at 30 °C on minimal medium described previously (Fulton et al., 1984), containing 0.2% (w/v) methanol, 0.2% (w/v) methanol and 0.2% (w/v) succinate, or 0.5% (v/v) methanol plus 0.2% (w/v) methylene. Antibiotics were added to the following final concentrations (mg l-1): tetracycline, 12.5; kanamycin, 25; and streptomycin, 10. Chemicals were obtained from Sigma. E. coli strains used for subcloning and sequence analyses were grown on L broth (Sambrook et al., 1989) containing 50 g ampicillin l-1. Nutrient agar and Bacto-agar were obtained from Difco.

**Bacterial matings and complementation analyses.** Tripertential matings between E. coli strains containing pHX200 derivatives and mobilizer plasmid and M. extorquens AM1 strains were performed overnight at 30 °C on nutrient agar plates. Exconjugates were then diluted and plated on minimal medium containing methanol, succinate or methylamine, the appropriate antibiotic solution, and rifampycin to select against E. coli.

**DNA manipulation.** Construction of subclones was performed following the methods of Sambrook et al. (1989), or as previously described by Morris et al. (1994) and Sambrook et al. (1992). DNA fragments for subcloning were purified using the Geneclean reagents (BiolOli). Enzymes were obtained from New England Biolabs and Boehringer Mannheim.

**Sequencing and sequence analysis.** The 6 kb HindIII-PstI fragment that complemented mxBM and mxBD mutants (Springer et al., 1993) was inserted into the sequencing vector pUC19 to make pCM191. The 4.5 kb BglII-PstI fragment from this plasmid was inserted into pUC19 to make pALS100 (Fig. 2). Subclones from these two clones were constructed using convenient restriction sites. Sequences were obtained from these subclones using the M13 forward and reverse primers designed for pUC19 (USB, New England Biolabs). Sequencing reactions were performed at the UCLA Sequencing Facility with an Applied Biosystems model 3730 automated sequencer. Sequence analyses were carried out using the Genetics Computer Group (GCG) program (Madison, WI, USA).

**Preparation of cell extracts.** Crude extracts of M. extorquens AM1 strains grown to late-exponential phase on 0.2% (w/v) succinate, 0.2% (w/v) methylamine, or 0.5% (v/v) methanol plus 0.2% (v/v) methylene were made using a French press as described by Chistoserdova & Lidstrom (1994). Protein concentrations were estimated spectrophotometrically (Whitaker & Granum, 1980).

**Catechol dioxygenase assays.** Activity of catechol 2,3-dioxygenase in the crude extracts was assayed at room temperature by adding 0.1 ml extract and 0.1 ml 10 mM catechol to 0.8 ml 50 mM Tris buffer solution (pH 7.5). Activity was monitored continuously for 15 s following addition of substrate with a Hewlett-Packard 8452A spectrophotometer. Catechol oxidation rates were determined using a molar extinction coefficient of 13800 (Kataeva & Golovleva, 1990).

## RESULTS

### Construction and analysis of an mxaF–xyE fusion in pHX200

It has previously been reported that a M. organophilum XX mxaF–xyE fusion was not expressed in MxBD and MxcQ mutants of M. extorquens AM1 (Xu et al., 1993).

![Fig. 1. Structure of pCMBam12. The direction of transcription for mxaF and xyE is indicated by large arrows. The transcriptional start site for mxaF is indicated by an asterisk and a small arrow. Abbreviations: MCS, multiple cloning site on pUC19; XbaI, BamHI; KpnI, SacI, BglII; B, BamHI; S, SalI.](image)

### Table 1. Expression of mxaF–xyE fusions in M. extorquens AM1 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity (nmol min⁻¹ (mg protein)⁻¹)</th>
<th>C₁</th>
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<tbody>
<tr>
<td></td>
<td>Succinate</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>30 ± 10</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>AM1</td>
<td>250 ± 30</td>
<td>3700 ± 2000</td>
</tr>
<tr>
<td>mxbD</td>
<td>20 ± 30</td>
<td>10 ± 10</td>
</tr>
<tr>
<td>mxbM</td>
<td>20 ± 30</td>
<td>10 ± 20</td>
</tr>
<tr>
<td>mxcQ</td>
<td>60 ± 30</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>mxcE</td>
<td>70 ± 50</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>mxdS</td>
<td>560 ± 150</td>
<td>3300 ± 300</td>
</tr>
</tbody>
</table>

To confirm the role of the mxb and mxc genes in expression of the M. extorquens AM1 mxaF, an mxaF–xyE fusion (pCMBam12) was constructed using a 1.56 kb Xhol–SalI fragment that extends 1.29 kb upstream of mxaF, and includes the transcription start site (Fig. 1; Morris & Lidstrom, 1992). Wild-type M. extorquens AM1 carrying pCMBam12 showed high expression of xyE when grown on C₁ compounds (methanol plus methylene; Table 1). This combination of C₁ substrates was used as the inducing condition because it results in a high level of MDH expression in both wild-type and methanol oxidation mutants (Nunn & Lidstrom, 1986a, b). The xyE expression in this strain was approximately 13-fold lower when it was grown on medium containing succinate as the carbon and energy source (Table 1). This change is comparable to the induction of MxaF.
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observed in immunoblots (Nunn & Lidstrom, 1986b), and suggests that this fusion construct accurately reports mxaF regulation.

pCMBam12 was introduced into mutant strains from four complementation groups: MxbD, MxbM, MxQ and MxE. The xylE activity observed for all of these strains was very low in cells grown either on C1 compounds or on succinate (Table 1). Two classes of non-regulatory mutants were also examined in this manner: MxdS (Table 1) and MxdR (data not shown). These strains showed expression of xylE that was similar to wild-type, indicating that the pattern observed for Mxb and Mx mutants was not a result of the inability to grow on methanol or of the inability to generate active MDH. For all classes of mutant, a negative control containing pHX200 was also examined. In all cases, these controls showed background levels of xylE expression (data not shown). These data confirm the importance of mxb and mx genes in transcription of the mxaF promoters, and suggest that no significant transcription occurred in either the succinate- or the C1-grown mutant strains.

**Nucleotide sequence of mxbD**

The region of DNA defining the MxbD and MxbM complementation groups indicated that mxbm extended across the left (5') BglII site as indicated in Fig. 2 (Springer et al., 1993). The nucleotide sequence to the right (5') of this site has already been reported, although the ORF corresponding to mxbM was not identified (Morris et al., 1994, GenBank accession no. L25889). The sequence reported here extends the known sequence from this BglII site 2.4 kb to the XmnI site as shown in Fig. 2. This region encodes all of mxbD and completes the sequence of mxbm.

The ORF corresponding to mxbD extends across the BamHI site (Fig. 2) as predicted from complementation analyses (Springer et al., 1993). A potential RBS is located five bases upstream of the putative translational start site. To confirm that this ORF corresponded to mxbD, a subclone was constructed that contained the predicted mxbD and no other intact ORFs. This subclone, pALS12 (Fig. 2), complemented MxbD mutants from M. extorquens AM1 as well as MxbD and MxbM mutants from M. organophilum XX. MxbM mutants were not complemented by pALS12. This confirmed that the predicted ORF was mxbD, and confirmed our previous report that the two M. organophili um XX complementation groups MxbN and MxbD are both complemented by mxbD in M. extorquens AM1 (Springer et al., 1995).

The putative protein encoded by mxbD is 545 amino acids, with a predicted molecular mass of 59 kDa (Fig. 3). MxbD shows homology to the family of bacterial histidine protein kinases (Stock et al., 1989). This homology is most significant over the C-terminal 280 amino acid region, which shows 20–30% amino acid identity to other kinases including EnvZ from E. coli (PIR accession no. B25024). The highest identity in this group is with CpxA, a multifunctional response regulator in E. coli (PIR accession no. S40855), which shares 30% amino acid identity over this region. The identity includes the five regions conserved in all histidine protein kinases (Stock et al., 1989; Parkinson & Kofoid, 1992): (I) His-308; (II) Asn-429; (III) DXGXG, residues 461–465; (IV) GXX, residues 489–91; and (V) Phe-474.
These regions are indicated in Fig. 3. Region I is the putative phosphorylation site (Stock et al., 1989), regions III and IV are thought to be involved in nucleotide binding (Parkinson & Kofoid, 1992). There are two putative transmembrane domains in the N-terminal half of MxbD (Fig. 3).

**Nucleotide sequence of mxbM**

The ORF corresponding to mxbM has a start codon that overlaps with the TGA stop codon of mxbD. The gene extends across the leftmost BglII site in Fig. 2, as predicted by complementation analyses (Springer et al., 1995). The putative mxbM ends 325 bases upstream of pqqD and has a predicted RBS 11 bases upstream of the ATG codon. The putative protein encoded by mxbM contains 235 amino acids and has a predicted molecular mass of 29 kDa. This protein has significant identity over its entire length to members of the family of aspartate kinase response regulators (Stock et al., 1989). The amino acid identity is 31−38% with other members of this family including E. coli OmpR and PhoB; and Streptomyces lividans CutR (PIR accession nos A25024, Rgexcfb, and S15274, respectively). The identity includes three conserved regions: (I) Asp-10, preceded by four hydrophobic amino acid residues 5−8; (II) Asp-53, preceded by four hydrophobic residues 49−52; and (III) Lys-103 (Stock et al., 1989). The conserved regions are indicated in Fig. 3. Region II is a putative phosphorylation site (Stock et al., 1989).

**Construction and analysis of mxbD-xylE fusions in pHX200**

To study the expression of mxbD, the 0.5 kb BamHI−XmnI fragment (Fig. 2) containing the first 331 bp of mxbD and 230 bp of upstream sequence, was inserted in front of the xylE reporter gene in the promoter probe vector pHX200 (Xu et al., 1993). This construct, pALS18 (Fig. 4), showed xylE expression in wild-type M. extorquens AM1 grown on C1 compounds (Table 2). The activity of xylE from pALS18 in cells grown on succinate was not significantly greater than background level.

To determine what portions of the upstream region were important for transcription, two more mxbD−xylE fusion constructs, pALS22 and pALS23, were made by PCR amplification of the insert in pALS18. These had deleted portions of the 5' end of the insert in pALS18 containing, respectively, 129 and 101 bp upstream of the translation start of mxbD (Fig. 4). To determine if elements further upstream of the promoter affected mxbD expression, pALS24 was constructed from the 0.9 kb SphI−BarnHI fragment (Fig. 2). This insert has the same 3' end as pALS18 but contains 675 bp upstream of mxbD (Fig. 4). The expression from these constructs is shown in Fig. 4. Neither of the deletion constructs showed any significant xylE expression in cells grown under inducing or non-inducing conditions. The xylE expression seen for pALS24 was similar to that seen for pALS18 under both conditions. These data suggest that a region of least 129 bases, and possibly as many as 229 bases, upstream of mxbD, is important for mxbD transcription in cells grown on C1 compounds.

**Effects of regulatory mutants on the mxbD−xylE fusions**

Activity of xylE was measured for pALS18 in different MxaB, Mxb and Mxc regulatory mutants (Table 3). MxaB, MxbD and MxbM mutants showed no detectable effect on expression, while MxcQ and Mxce mutants showed greatly reduced expression of the mxbD−xylE fusion in cells grown on C1 compounds. A non-regulatory mutant, mxaF, also showed no effect on xylE expression. In all strains tested, xylE expression in

**Table 2. Expression of mxbD−xylE fusions in wild-type M. extorquens AM1**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Activity [nmol min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate</td>
</tr>
<tr>
<td>pALS24 (675 bp)</td>
<td>40 ± 40</td>
</tr>
<tr>
<td>pALS18 (299 bp)</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>pALS23 (128 bp)</td>
<td>10 ± 10</td>
</tr>
<tr>
<td>pALS22 (101 bp)</td>
<td>10 ± 10</td>
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</tbody>
</table>
Table 3. Expression from pALS18, the 0.6 kb insert mxbD-xylE fusion, in different regulatory mutant strains of M. extorquens AM1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>Succinate</th>
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<tr>
<td>Control</td>
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<tr>
<td>AM1</td>
<td>20 ± 10</td>
<td>140 ± 30</td>
<td></td>
</tr>
<tr>
<td>mxB</td>
<td>40 ± 20</td>
<td>110 ± 40</td>
<td></td>
</tr>
<tr>
<td>mXB</td>
<td>30 ± 10</td>
<td>100 ± 30</td>
<td></td>
</tr>
<tr>
<td>mxE</td>
<td>20 ± 10</td>
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<td></td>
</tr>
<tr>
<td>mxaF</td>
<td>30 ± 10</td>
<td>130 ± 10</td>
<td></td>
</tr>
</tbody>
</table>

Activity of xylE is given as catechol 2,3-dioxygenase activity in crude extracts. Cells were grown on 0.2% (w/v) succinate or, for C₁ growth, on 0.2% (w/v) methanolamine plus 0.5% (v/v) methanol. All values were determined at least twice, and values given indicate their range.

cells grown on succinate was at background levels. The effects of the regulatory mutants on mxbD-xylE expression in pALS24 was the same as that seen for pALS18 (data not shown). For all mutant strains, the xylE expression from a negative control containing pHX200 was also examined. In all cases, these controls showed background levels of xylE expression similar to that seen in wild-type containing pHX200 (data not shown).

DISCUSSION

The sequence data presented here suggest that MxbD belongs to the family of bacterial histidine protein kinases (Stock et al., 1989). The putative amino acid sequence suggests that the C-terminal portion of the protein has a kinase domain and that the N-terminal portion contains two transmembrane regions. MxbD does not contain an aspartate phosphorylation domain and so it is apparently not a hybrid kinase (Swanson et al., 1994). The region between the putative transmembrane regions (approximately residues 30-190) may be periplasmic. Such a region could function as a sensor, receiving a signal which is then transduced to MxbM. The signal sensed by MxbD is not known. MxbM shows homology throughout its length to other bacterial response regulators (Stock et al., 1989). The subfamily to which MxbM shows the best identity are all DNA-binding proteins, suggesting that MxbM may have a DNA-binding region in its C-terminal half (Parkinson & Kofoid, 1992).

The ORFs of mxbD and mxbM overlap at their respective stop and start codons, indicating that they are probably co-transcribed. No ORF was identified that ended near the start of mxbD, and promoter activity was found within 229 bp of the translational start codon, suggesting that mxbD is located at the beginning of the transcript. The genes downstream of mxbDm, pqqDCG (Fig. 2), are apparently transcribed separately from mxbD. A gap of 325 bp exists between mxbM and pqqD, and a transcriptional start site for pqqD has been identified within this region (Ramamoorthi & Lidstrom, 1995).

The mxbD-xylE fusions showed that the 229 bp region upstream of the translation start of mxbD contains sufficient information to allow transcription under inducing conditions. The analysis of nested deletions of the S′ end of this region suggests that elements within the stretch from −229 to −129 upstream of the mxbD start are required for transcription. Scanning of this sequence did not reveal any significant direct or inverted repeats. However, a potential σ⁺ −10 sequence was identified centred 33 bp upstream of the translation start site (Fig. 5), and a potential −35 sequence is centred 17 bp further upstream. Another region of interest is the GAAA sequence at about −80. GAAA sequences are common upstream of methylotrophic promoters, although their significance is not known (Lidstrom & Stirling, 1990). The putative −35 and −10 sequences of the mxaF promoter (Lidstrom & Stirling, 1990) are not seen in this region, but since the mxbD promoter has lower activity than that of mxaF it is likely that the −35 and −10 sequences will be different. The mxbD promoter is also regulated differently than the mxaF promoter, since it is affected only by Mxc mutants, not by MxB or Mxb mutants.

The results presented here together with previous data demonstrate that in the Methylobacterium strains, at least two separate sensor kinase–response regulator pairs are involved in transcriptional regulation of methanol oxidation genes. In the current study we have shown that in M. extorquens AM1, one of these pairs (MxcQE) controls expression of the second pair (MxbDM). Since these genes are interchangeable between the Methylobacterium strains (Bastien et al., 1989; Xu et al., 1993; Springer et al., 1995), it is likely that MxcQE also control expression of MxbDM in M. organophilum XX. In these Methylobacterium strains, this second sensor kinase–response regulator pair (MxbDM) is required for normal expression of mxaF.
Both of the proposed sensor kinases (MxbD and MxcQ) are involved in the negative regulation of expression of cytochrome c_{553} (Springer et al., 1995). However, MxcQE are not required for mxaW or pqqD expression. Therefore, low level expression of mxbDM must occur in the mxcQE mutants, below the level of detection by the xyle fusion system, which is sufficient for transcription of pqqD and mxaW, but is not sufficient for transcription of mxaF, nor for repression of cytochrome c_{553}.

These data suggest a hierarchy of regulation in which MxcQE control the level of expression of MxbDM, which in turn control expression of other genes involved in methanol oxidation. However, it is not known whether MxcE may in addition play a direct role in mxaF transcription. In M. organophilum XX, extracts from an MxcQ mutant do not cause a gel shift observed in the wild-type, of a DNA fragment containing both the mxaF and the mxaW promoters (Xu et al., 1995). These data suggest that MxcQ is required for the production of a DNA-binding protein that binds to this region, but the identity of that protein is unknown. Similar experiments with an MxcE mutant were less conclusive, showing a decreased gel shift response. Again, in this case it is not clear if MxcE binds to this DNA fragment, or whether it is required for expression of a different protein that acts downstream in the regulatory scheme. In addition, a third regulatory gene, mxaB, is also required for normal expression of mxaF, pqqD and cytochrome c_{553} but not mxaW. The role of this protein in the methanol oxidation regulatory hierarchy is also not known, except that this study has shown it is not involved in expression of mxbDM.

Both of the proposed sensor kinases (MxbD and MxcQ) are predicted to contain periplasmic loops, but these do not show identity with each other (this study; Xu et al., 1995). These results suggest that at least two different signals may be involved in regulation of methanol oxidation genes, and that these are probably sensed in the periplasm. The identity of the compounds that are sensed is unknown, but obvious candidates present in the periplasm are methanol, formaldehyde and PQP.

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to synthesize pyrroloquinoline quinone and sequences of pqqD, pqqG and pqqC. 


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