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

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Five genes are thought to be required for transcription of methanol oxidation genes in *Methylobacterium* strains. These putative regulatory genes include *mxsQE*, which encode a putative sensor–regulator pair, and *mxsDM* and *mxsB*, whose functions are less well-understood. In this study, *mxsDM* in *Methylobacterium extorquens* AM1 were shown to be required for expression of a *xylE* transcriptional fusion to the structural gene for the large subunit of methanol dehydrogenase (*mxsF*), confirming the role of these genes in transcriptional regulation of *mxsF*. The nucleotide sequence suggests that *mxsD* encodes a histidine protein kinase with two transmembrane domains and that *mxsM* encodes a DNA-binding response regulator. A *xylE* transcriptional fusion to the putative *mxsD* 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 *mxsD* is required for expression. The expression of the *mxsD*–*xylE* fusion was examined in each of the five known regulatory mutant classes. *xylE* expression was reduced to non-detectable levels in MxsQ and MxsE 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 MxsQE control expression of the sensor–regulator pair MxsDM, and MxsDM in turn control expression of a number of genes involved in methanol oxidation.

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

INTRODUCTION

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 *et al.*, 1992; Richardson & Anthony, 1992; White *et al.*, 1993; Anthony *et al.*, 1994; Ghosh *et al.*, 1995).

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

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

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genes: *mxafJGIRSACKLDB* (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 *mxal*, respectively, and *mxag* encodes the associated cytochrome c_L electron acceptor (Nunn & Lidstrom, 1986a, b; Anderson & Lidstrom, 1988; Nunn & Anthony, 1988). At least four of these genes, *mxackL*, 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 *mxqE*, 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 (Laufer & Lidstrom, 1992; Morris & Lidstrom, 1992). Other C_1 compounds such as methylamine will also induce MDH, even though MDH is not required for growth on these other substrates (Laufer & 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 *mxq* genes may also be involved in regulation of a novel c -type cytochrome, cytochrome c_{553} , which is present at elevated levels in *mxb* and *mxq* 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 methylophilic. *mxq* and *mxqE* 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 *Methylobacterium* 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 *MxcQE*, 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*, EMS52. *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 *Bam*HI–*Xmn*I fragment (Fig. 2), which was cloned into pUC19 to make pALS122. The insert was then cut out with *Hind*III and *Bam*HI 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 *Xba*I and *Bam*HI and inserted into those sites of pHX200. pALS24 was derived from a 0.8 kb *Sph*I–*Bam*HI fragment (Fig. 2) in pALS128. This insert was cut out with *Hind*III and *Bam*HI and cloned into those sites in the promoter probe vector pHX200. The *mxaf-xylE* fusion pCMBam12 was made using the 1.55 kb *Xho*I–*Sal*I fragment from a 410XSP7 (Morris & Lidstrom, 1992), which was cloned into the *Sal*I site of pUC7 (Vieira & Messing, 1982), cut out with *Bam*HI, and inserted into the *Bam*HI site of pHX200 to make pCMBam12 (Fig. 1). pALS12 was a derivative of pRK310 (Tc^R , IncPI; Ditta *et al.*, 1985). The mobilizing plasmid used for conjugation was pRK2073 (Sm^R ; Figurski & Helinski, 1979). *E. coli* strain DH5 α (BRL) was used for DNA

manipulation. Plasmids used for sequencing analysis were derivatives of vector pUC19 (Yanisch-Perron *et al.*, 1985).

Media and growth conditions. *Methylobacterium* strains were grown at 30 °C on minimal medium described previously (Fulton *et al.*, 1984), containing 0.2% (v/v) methanol, 0.2% (w/v) methylamine and 0.2% (w/v) succinate, or 0.5% (v/v) methanol plus 0.2% (w/v) methylamine. 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. Triparental 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 rifamycin to select against *E. coli*.

DNA manipulation. Construction of subclones was performed following the methods of Sambrook *et al.* (1989), or as described previously (Morris *et al.*, 1994; Morris & Lidstrom, 1992). DNA fragments for subcloning were purified using the GeneClean reagents (Bio101). 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.*, 1995) 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 373A 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% (w/v) methanol plus 0.2% (v/v) methylamine 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). A₃₇₆ 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 13 800 (Kataeva & Golovleva, 1990).

RESULTS

Construction and analysis of an *mxoF*–*xylE* fusion in pHX200

It has previously been reported that a *M. organophilum* XX *mxoF*–*xylE* 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 *mxoF*' and *xylE* is indicated by large arrows. The transcriptional start site for *mxoF*' is indicated by an asterisk and a small arrow. Abbreviations: MCS, multiple cloning site on pHX200 (*HindIII*, *XbaI*, *BamHI*, *KpnI*, *SacI*, *BglII*); B, *BamHI*; S, *SalI*.

Table 1. Expression of *mxoF*–*xylE* fusions in *M. extorquens* AM1 strains

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) methylamine plus 0.5% (v/v) methanol. Control, pHX200 with no promoter (in wild-type cells). Other strains are pCMBam12 in wild-type *M. extorquens* AM1 and mutants as indicated (see Methods). All values were determined at least twice, and values given indicate their range.

Strain	Activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	Succinate	C ₁
Control	30 ± 10	10 ± 10
AM1	250 ± 30	3700 ± 2000
<i>mxoD</i>	20 ± 30	10 ± 10
<i>mxoM</i>	20 ± 30	10 ± 20
<i>mxoQ</i>	60 ± 30	30 ± 10
<i>mxoE</i>	70 ± 50	20 ± 10
<i>mxoS</i>	560 ± 150	3300 ± 300

To confirm the role of the *mxo* and *mxo* genes in expression of the *M. extorquens* AM1 *mxoF*, an *mxoF*–*xylE* fusion (pCMBam12) was constructed using a 1.56 kb *XhoI*–*SalI* fragment that extends 1.29 kb upstream of *mxoF*, and includes the transcription start site (Fig. 1; Morris & Lidstrom, 1992). Wild-type *M. extorquens* AM1 carrying pCMBam12 showed high expression of *xylE* when grown on C₁ compounds (methanol plus methylamine; 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 *xylE* 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 *MxoF*

Fig. 2. Restriction map of the Mxb region. The hatched line indicates the sequence reported in this study. The thick black line indicates the region that was sequenced previously (Morris *et al.*, 1994). The ORFs are indicated as large arrows, with gene designations underneath. Clones used for sequencing or complementation analysis are indicated. Abbreviations: B, *Bam*HI; G, *Bgl*II; H, *Hind*III; P, *Pst*I; S, *Sal*I; Xm, *Xmn*I.

observed in immunoblots (Nunn & Lidstrom, 1986b), and suggests that this fusion construct accurately reports *mxhF* regulation.

pCMBam12 was introduced into mutant strains from four complementation groups: MxbD, MxbM, MxcQ and MxcE. The *xylE* activity observed for all of these strains was very low in cells grown either on C_1 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 Mxc 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 *mxh* and *mxh* genes in transcription of the *mxhF* promoters, and suggest that no significant transcription occurred in either the succinate- or the C_1 -grown mutant strains.

Nucleotide sequence of *mxhD*

The region of DNA defining the MxbD and MxbM complementation groups indicated that *mxhM* extended across the left (3') *Bgl*II site as indicated in Fig. 2 (Springer *et al.*, 1995). The nucleotide sequence to the right (5') of this site has already been reported, although the ORF corresponding to *mxhM* was not identified (Morris *et al.*, 1994, GenBank accession no. L25889). The sequence reported here extends the known sequence from this *Bgl*II site 2.4 kb to the *Xmn*I site as shown in Fig. 2. This region encodes all of *mxhD* and completes the sequence of *mxhM*.

The ORF corresponding to *mxhD* extends across the *Bam*HI site (Fig. 2) as predicted from complementation analyses (Springer *et al.*, 1995). A potential RBS is located five bases upstream of the putative translational

MxbD

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1  MRSRLRVFAL LLGGTALIAL LAATGVLSI RLTVNVIERT LAQHRLELL
51  AELSGRLAQY GFAAIDSVGN PDAPPEALSV TRDRVDQALN HVDDAIGSGM
101  ATSANLEDRI QYVARTPLA NVRAARMLD RQVTLVRRET DPLDRKTAMQ
151  GALNAPGAMT GPPLSLLEIA ERRSMTAGSE AARTLSQRLT AAALAATVLA
201  LAFTVGVLYRS VTRPTLARI EIRRAAGAVG SGALGTRLSV ETRDELGLLV
251  ANFNRMARL ARREGRLAD RAAEDTVEA RTADLRAANG ALEMVDRSR
301  RFFADVSEL RTPLTVILGE CDLAQRSAEA AAGRMVDYGP VFTTIRKRAQ
351  RLKRRVEDLF AWPARNPARS SSTGATVSAV EVLTDVSDA GSEAARRRVE
401  LVLEPGDHI EIAADPEWL QVVESLIDN LRHATGLTRI TVSLSEHDTF
451  QGRRVWTTID DDFGFPAGG DGLFERFRE ARSGEAGFET LALARWVVE
501  RHDGAISLKG AEHGGGARVV IDLPSLDGV APESYGAGHE RIGAA

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MxbM

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1  MTTRVLIVE DIDIRGILAR GLEAEFVSIG VAGRVEDALS AARDEAPEAV
51  VLITLPGS GHDVCRSLRE GGYPGAILFL SARDEVDRRA EGLALGADDY
101  IVDFVFDL LARLQVHLLR RREADTPRTV FTAGRLTLDV NIRQVSFGEA
151  SARLTPREAE LLALLMQEVN RPISRGEIFD RLWASQGLS LNVVDVYGVY
201  LRSKLSDFVR LGGPVIVTVR KGKFMGLDRG QDFRH

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Fig. 3. The amino acid sequences of MxbD and MxbM. For MxbD, the putative transmembrane regions are underlined, conserved regions I–V (see text) are indicated with boxes. For MxbM, the conserved regions I–III (see text) are indicated with boxes.

start site. To confirm that this ORF corresponded to *mxhD*, a subclone was constructed that contained the predicted *mxhD* and no other intact ORFs. This subclone, pALS12 (Fig. 2), complemented MxbD mutants from *M. extorquens* AM1 as well as MxbD and MxbN mutants from *M. organophilum* XX. MxbM mutants were not complemented by pALS12. This confirmed that the predicted ORF was *mxhD*, and confirmed our previous report that the two *M. organophilum* XX complementation groups MxbN and MxbD are both complemented by *mxhD* in *M. extorquens* AM1 (Springer *et al.*, 1995).

The putative protein encoded by *mxhD* 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) GXG, residues 489–91; and (V) Phe-474.

Fig. 4. Structure of *mxbD*-*xylE* fusions. Direction of transcription of *mxbD'* and *xylE* is indicated by large arrows. Abbreviations: MCS, multiple cloning site on pHX200 (*Hind*III, *Xba*I, *Bam*HI, *Kpn*I, *Sac*I, *Bgl*II); B, *Bam*HI; H, *Hind*III; Sp, *Sph*I.

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 & Kofoed, 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 *Bgl*II 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, Rgecfb, 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 *Bam*HI-*Xmn*I fragment (Fig. 2) containing the first 331 bp of *mxbD* and 230 bp of upstream sequence, was inserted in

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

The length of the region upstream of *mxbD* is indicated in parentheses for each fusion. Control, pHX200 with no promoter (in wild-type cells). 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) methylamine plus 0.5 % (v/v) methanol. All values were determined at least twice, and values given indicate their range.

Plasmid	Activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	Succinate	C ₁
Control	30 ± 10	10 ± 10
pALS24 (675 bp)	40 ± 40	160 ± 30
pALS18 (229 bp)	20 ± 10	140 ± 30
pALS23 (128 bp)	10 ± 10	20 ± 10
pALS22 (101 bp)	10 ± 10	20 ± 10

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 *Sph*I-*Bam*HI 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 at 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, *mxoF*, also showed no effect on *xylE* expression. In all strains tested, *xylE* expression in

Table 3. Expression from pALS18, the 0.6 kb insert *mxbD*-*xylE* fusion, in different regulatory mutant strains of *M. extorquens* AM1

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) methylamine plus 0.5% (v/v) methanol. All values were determined at least twice, and values given indicate their range.

Strain	Activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	Succinate	C ₁
Control	30 ± 10	10 ± 10
AM1	20 ± 10	140 ± 30
<i>mxsA</i> B	40 ± 20	110 ± 40
<i>mxsB</i> D	30 ± 10	100 ± 30
<i>mxsB</i> M	30 ± 10	100 ± 20
<i>mxsC</i> Q	20 ± 10	20 ± 10
<i>mxsC</i> E	20 ± 10	20 ± 10
<i>mxsA</i> F	30 ± 10	130 ± 10

cells grown on succinate was at background levels. The effects of the regulatory mutants on *mxsB*D-*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 *mxsB*D and *mxsB*M 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 *mxsB*D, and promoter activity was found within 229 bp of the translational start codon, suggesting that *mxsB*D is located at the beginning

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[ XmnI
-229 GAACCCCTTC CTGTGTCGTC GCAACTCGCA GAGCAGCTC GCGGCGTGCC
-179 AGGAGGAGCA TCCGGACTAT CACGTGAAGA GTTGCGCTG CGCCCCAAG
-129 ] GCGAGCCTAC CGACCGACCT GTAGGACCA CCTGCGAGCG GACCGGAAA
      A5120 A590
      σ70-35 σ70-10
-79 AGCCGGCAAT GCGAGCCGGC TTGAGACCT GCCCGGCGG TGA CATGGTC
      Start mxbD
-29 CGCGCGCCGC CACTGCGGAG AGGGAGACCA TCGTTCGCT GAGAGTCGG
      Met-Arg-Ser-Lys-Arg-Val-Arg

```

Fig. 5. Sequence upstream of *mxsB*D. Putative σ^{70} -10 and -35 sequences described in the text are indicated by underlining, and sequences used for the A590 and A5120 primers are indicated by open boxes. The -229 to -129 region is indicated by brackets.

of the transcript. The genes downstream of *mxsB*DM, *pqqDGC* (Fig. 2), are apparently transcribed separately from *mxsB*DM. A gap of 325 bp exists between *mxsB*M and *pqqD*, and a transcriptional start site for *pqqD* has been identified within this region (Ramamoorthi & Lidstrom, 1995).

The *mxsB*D-*xylE* fusions showed that the 229 bp region upstream of the translation start of *mxsB*D contains sufficient information to allow transcription under inducing conditions. The analysis of nested deletions of the 5' end of this region suggests that elements within the stretch from -229 to -129 upstream of the *mxsB*D start are required for transcription. Scanning of this sequence did not reveal any significant direct or inverted repeats. However, a potential σ^{70} -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 *mxsA*F promoter (Lidstrom & Stirling, 1990) are not seen in this region, but since the *mxsB*D promoter has lower activity than that of *mxsA*F, it is likely that the -35 and -10 sequences will be different. The *mxsB*D promoter is also regulated differently than the *mxsA*F 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 (*mxsB*DM). 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 *mxsB*DM in *M. organophilum* XX. In these *Methylobacterium* strains, this second sensor kinase-response regulator pair (*mxsB*DM) is required for normal expression of *mxsA*F

(Xu *et al.*, 1993), *pqqD* (Ramamoorthi & Lidstrom, 1995) and *mxw* (Xu *et al.*, 1993), and is also apparently involved in the negative regulation of expression of cytochrome *c₅₅₃* (Springer *et al.*, 1995). However, MxcQE are not required for *mxw* 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 *mxw*, but is not sufficient for transcription of *mxwF*, nor for repression of cytochrome *c₅₅₃*.

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 MxcQE may in addition play a direct role in *mxwF* 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 *mxwF* and the *mxw* 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, *mxwB*, is also required for normal expression of *mxwF*, *pqqD* and cytochrome *c₅₅₃*, but not *mxw*. 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 PQQ.

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REFERENCES

- Anderson, D. J. & Lidstrom, M. E. (1988). The *moxFG* region encodes four polypeptides in the methanol-oxidizing bacterium *Methylobacterium* sp. strain AM1. *J Bacteriol* **170**, 2254–2262.
- Anthony, C. (1986). Bacterial oxidation of methane and methanol. *Adv Microb Physiol* **27**, 113–210.
- Anthony, C. (1992). The *c*-type cytochromes of methylophilic bacteria. *Biochim Biophys Acta* **1099**, 1–15.

- Anthony, C., Ghosh, M. & Blake, C. C. F. (1994). The structure and function of methanol dehydrogenase and related PQQ-containing quinoproteins. *Biochem J* **304**, 665–674.
- Bastien, C., Machlin, S., Zhang, Y., Donaldson, K. & Hanson, R. S. (1989). Organization of genes required for the oxidation of methanol to formaldehyde in three type II methylotrophs. *Appl Environ Microbiol* **55**, 3124–3130.
- Chistoserdova, L. V. & Lidstrom, M. E. (1994). Genetics of the serine cycle in *Methylobacterium extorquens* AM1: identification of *sgaA* and *mtaA* and sequences of *sgaA*, *hprA* and *mtaA*. *J Bacteriol* **176**, 1957–1968.
- Day, D. J., Nunn, D. N. & Anthony, C. (1990). Characterization of a novel soluble *c*-type cytochrome in a *moxD* mutant of *Methylobacterium extorquens* AM1. *J Gen Microbiol* **136**, 181–188.
- Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.-W., Finlay, D. R., Guiney, D. & Helinski, D. R. (1985). Plasmids related to the broad host range vector pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13**, 149–153.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* **76**, 1648–1652.
- Fulton, G. L., Nunn, D. N. & Lidstrom, M. E. (1984). Molecular cloning of a malyl CoA lyase gene from *Pseudomonas* AM1, a facultative methylotroph. *J Bacteriol* **160**, 718–723.
- Ghosh, M., Anthony, C., Harlos, K., Goodwin, M. G. & Blake, C. C. F. (1995). The refined structure of the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens* at 1.94 Å. *Structure* **3**, 177–187.
- Harms, N., Reijnders, W. N. M., Anazawa, H. & Van der Palen, C. H. N. M. (1993). Identification of a two component regulatory system controlling methanol dehydrogenase in *Paracoccus denitrificans*. *Mol Microbiol* **8**, 457–470.
- Kataeva, I. A. & Golovleva, L. A. (1990). Catechol 2,3-dioxygenases from *Pseudomonas aeruginosa* 2x. *Methods Enzymol* **188**, 115–121.
- Laufer, K. & Lidstrom, M. E. (1992). Regulation and expression of bacterial quinoproteins. In *Quinoproteins*, pp. 193–222. Edited by V. Davidson. New York: Marcel Dekker.
- Lee, K. E., Stone, S., Goodwin, P. M. & Holloway, B. W. (1991). Characterization of transposon insertion mutants of *Methylobacterium extorquens* AM1 (*Methylobacterium* strain AM1) which are defective in methanol oxidation. *J Gen Microbiol* **137**, 895–904.
- Lidstrom, M. E. & Stirling, D. I. (1990). Methylotrophs: genetics and commercial applications. *Annu Rev Microbiol* **44**, 27–58.
- Lidstrom, M. E., Anthony, C., Biville, F., Gasser, F., Goodwin, P., Hanson, R. S. & Harms, N. (1994). New unified nomenclature for genes involved in the oxidation of methanol in Gram-negative bacteria. *FEMS Microbiol Lett* **117**, 103–106.
- Machlin, S. M., Tam, P. E., Bastien, C. A. & Hanson, R. S. (1987). Genetic and physical analyses of *Methylobacterium organophilum* XX genes encoding methanol oxidation. *J Bacteriol* **170**, 141–148.
- Morris, C. J. & Lidstrom, M. E. (1992). Cloning of a methanol-inducible *moxF* promoter and its analysis in *moxB* mutants of *Methylobacterium extorquens* AM1. *J Bacteriol* **174**, 4444–4449.
- Morris, C. J., Biville, F., Turlin, E., Lee, E., Ellermann, K., Fan, W.-H., Ramamoorthi, R., Springer, A. L. & Lidstrom, M. E. (1994). Isolation, phenotypic characterization and complementation analysis of mutants of *Methylobacterium extorquens* AM1 unable

- to synthesize pyrroloquinoline quinone and sequences of *pqqD*, *pqqG* and *pqqC*. *J Bacteriol* **176**, 1746–1755.
- Morris, C. J., Springer, A. L., Kim, Y. M., Perkins, K. E. & Lidstrom, M. E. (1995). The identification and nucleotide sequence of *mxmA*, *mxnC*, *mxnK*, *mxnL* and *mxnD* genes from *Methylobacterium extorquens* AM1. *J Bacteriol* **177**, 6825–6831.
- Nunn, D. N. & Anthony, C. (1988). The nucleotide sequence and deduced amino acid sequence of the cytochrome *c_L* gene of *Methylobacterium extorquens* AM1, a novel class of *c*-type cytochrome. *Biochem J* **256**, 673–676.
- Nunn, D. N. & Lidstrom, M. E. (1986a). Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. *J Bacteriol* **166**, 581–590.
- Nunn, D. N. & Lidstrom, M. E. (1986b). Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylobacterium extorquens* sp. strain AM1. *J Bacteriol* **166**, 591–597.
- Parkinson, J. S. & Kofoed, E. C. (1992). Communication modules in bacterial signaling proteins. *Annu Rev Genet* **26**, 71–112.
- Ramamoorthi, R. & Lidstrom, M. E. (1995). Transcriptional analysis of *pqqD* and study of the regulation of pyrroloquinoline quinone biosynthesis of *Methylobacterium extorquens* AM1. *J Bacteriol* **177**, 206–211.
- Richardson, I. W. & Anthony, C. (1992). Characterization of mutant forms of the quinoprotein methanol dehydrogenase lacking an essential calcium ion. *Biochem J* **287**, 709–715.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- van Spanning, R. J. M., Wansell, C. W., De Boer, T., Hazelaar, M. J., Anazawa, H., Harms, N., Oltmann, L. F. & Stouthamer, A. H. (1991). Isolation and characterization of the *moxJ*, *moxG*, *moxI* and *moxR* genes of *Paracoccus denitrificans*: inactivation of *moxJ*, *moxG* and *moxR* and the resultant effect on methylo-trophic growth. *J Bacteriol* **173**, 6948–6961.
- Springer, A. L., Chou, H.-H., Fan, W.-H., Lee, E. & Lidstrom, M. E. (1995). Methanol oxidation mutants in *Methylobacterium extorquens* AM1: identification of new genetic complementation groups. *Microbiology* **141**, 2985–2993.
- Stock, J. B., Ninfa, A. J. & Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev* **53**, 450–490.
- Swanson, R. V., Alex, L. A. & Simon, M. I. (1994). Histidine and aspartate phosphorylation: two component systems and the limits of homology. *Trends Biochem Sci* **19**, 485–490.
- Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- Whitaker, J. R. & Granum, P. E. (1980). An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal Biochem* **109**, 156–159.
- White, S., Boyd, G., Mathews, F. S., Xia, Z.-X., Dai, W.-W., Zhang, Y.-F. & Davidson, V. L. (1993). The active site structure of the calcium-containing quinoprotein methanol dehydrogenase. *Biochemistry* **32**, 12955–12958.
- Xia, Z.-X., Dai, W.-W., Xiong, J.-P., Davidson, V. L., White, S. & Mathews, F. S. (1992). The three-dimensional structures of methanol dehydrogenase from two methylotrophic bacteria at 2.6 Å resolution. *J Biol Chem* **267**, 22289–22297.
- Xu, H. H., Viebahn, M. & Hanson, R. S. (1993). Identification of methanol-regulated promoter sequences from the facultative methylotrophic bacterium *Methylobacterium organophilum* XX. *J Gen Microbiol* **139**, 743–752.
- Xu, H. H., Janka, J. J., Viebahn, M. & Hanson, R. S. (1995). Nucleotide sequence of the *mxcQ* and *mxcE* genes, required for methanol dehydrogenase synthesis in *Methylobacterium organophilum* XX: a two-component regulatory system. *Microbiology* **141**, 2543–2551.
- Yang, H., Reijnders, W. N. M., van Spanning, R. J. M., Stout-hamer, A. H. & Harms, N. (1995). Expression of the structural *mox* genes in *Paracoccus denitrificans* follows wild-type regulation in mutants with a deletion in *mxnY*, the gene encoding the signal sensor. *Microbiology* **141**, 825–830.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

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