Nucleotide sequence of the *mxoQ* and *mxoE* genes, required for methanol dehydrogenase synthesis in *Methylobacterium organophilum* XX: a two-component regulatory system

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Nucleotide sequence analysis of the *mxoQ* and *mxoE* loci, required for the synthesis of methanol dehydrogenase in *Methylobacterium organophilum* XX, has revealed two open reading frames that show significant similarity to sequences of prokaryotic two-component systems, especially MxaY and MxaX proteins of another methylotrophic bacterium, *Paracoccus denitrificans*. Cell-free extracts and DNA-column-fractionated proteins from wild-type *M. organophilum* XX cells grown on methanol or succinate contained protein(s) that were able to bind specifically to the upstream region of methanol dehydrogenase large subunit gene (*mxoF*). In contrast, cell-free extracts from *mxoQ* and *mxoE* mutant strains of *M. organophilum* XX had zero or reduced binding activity towards the promoter fragments of the *mxoF* gene. This is consistent with the involvement of the *mxoQ* and *mxoE* genes in transcriptional regulation of methanol dehydrogenase synthesis. Analyses of sequential deletions of the *mxoF* upstream region have defined the functional boundary of the promoter/operator region of this gene and identified one nucleotide segment as essential to the activation of *mxoF*.

Keywords: methylotrophs, *Methylobacterium organophilum* XX, two-component system, methanol dehydrogenase

INTRODUCTION

Methylobacterium organophilum XX is a pink-pigmented, facultative, type II methylotrophic bacterium (Patt *et al.*, 1976). The enzymes involved in growth of some facultative methylotrophs on one-carbon compounds can be induced by methanol or methylamine (Anthony, 1982; O'Connor & Hanson, 1977). The oxidation of methanol to formaldehyde is catalysed by methanol dehydrogenase (MDH). This is a periplasmic enzyme and contains the prosthetic group pyrroloquinoline quinone (PQQ). This enzyme is abundant in all of the Gram-negative methylotrophic bacteria thus far examined (Anthony, 1982). The

MDH enzymes of Gram-negative methylotrophs are tetramers of two types of subunits in an $\alpha_2\beta_2$ conformation (Anthony, 1982, 1986; Cox *et al.*, 1992; Nunn *et al.*, 1989). The large subunit (α subunit) has a molecular mass of 56–76 kDa, while the small subunit (β subunit) has a molecular mass of 8–10 kDa (Nunn *et al.*, 1989; Cox *et al.*, 1992; Anderson *et al.*, 1990). The structural gene for the large subunit of MDH [*mxoF*, formerly *moxF* (Lidstrom *et al.*, 1994)] from *M. organophilum* XX has been cloned and sequenced, and its promoter and transcriptional start sites have been identified (Machlin & Hanson, 1988). Genetics studies of MDH synthesis in *M. organophilum* XX (Allen & Hanson, 1985; Machlin *et al.*, 1988; Machlin & Hanson, 1988; Bastien *et al.*, 1989) indicated that seven separate chromosomal regions (groups I–VII) encode a minimum of 15 genes that are required for methanol oxidation and formaldehyde assimilation. Among these genes, *mxoQ* and *mxoE* [formerly *moxQ* and *moxE* (Lidstrom *et al.*, 1994)] have been found to be involved in the regulation of synthesis of MDH (Machlin *et al.*, 1988; Bastien *et al.*, 1989).

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Abbreviation: MDH, methanol dehydrogenase.

The GenBank accession number for the sequence reported in this paper is U18290.

Recently, we reported the construction of a promoter-probe vector for methylotrophic bacteria and its application in identifying methanol-regulated promoter fragments (Xu *et al.*, 1993). This vector was also used to analyse *mxoF* promoter activity *in vivo* in *mxoQ* and *mxoE* mutant strains of *M. organophilum* and further confirmed the involvement of these two genes in transcriptional regulation of *mxoF* gene (Xu *et al.*, 1993).

METHODS

Media and growth conditions. *Escherichia coli* JM109 was grown in Luria-Bertani medium at 37 °C. *Methylobacterium organophilum* XX was grown in MacLennan minimal medium (MM) (MacLennan *et al.*, 1971) containing 0.5% (v/v) methanol or 0.1% succinate at 30 °C. When required, antibiotics were added at the following final concentrations: 50 µg ml⁻¹ of ampicillin (Ap) and kanamycin (Km) for *E. coli* strains; 15 µg ml⁻¹ and 20 µg ml⁻¹ of tetracycline (Tc) for *E. coli* and *M. organophilum* XX, respectively.

DNA manipulation. Plasmid isolation and purification, restriction endonuclease digestion, ligation and DNA dephosphorylation were performed as described by Sambrook *et al.* (1989). Competent cells of *E. coli* JM109 were transformed with various plasmid constructs based on the protocols provided by Promega.

Cloning and sequence analysis of *mxoQ* and *mxoE* genes. A 5.5 kb *EcoRI* restriction fragment containing *mxoQ* and *mxoE* (Machlin *et al.*, 1988) was cloned into pBluescript II SK(+) (Stratagene). From this plasmid (pBS-C6), a 2.5 kb *BamHI*-*BglII* restriction fragment was excised, resulting in a plasmid (pBS-C6Δ2.5) with about 3.0 kb DNA insert which primarily contains *mxoQ* and *mxoE*. In order to sequence this insert, five smaller fragments were further cloned into pBluescript II SK(+). Double-stranded plasmid DNA was directly used for sequencing based on protocols provided with the Sequenase Version 2.0 Sequencing Kit by US Biochemicals. Compressions were resolved by using deazanucleotide analogues in the reaction mixtures. Fourteen oligonucleotides, designed from available nucleotide sequence and synthesized by the Microchemical Facility, University of Minnesota, were used to complete the sequencing of the whole region from both DNA strands. Sequence data were analysed with the help of the University of Wisconsin Genetics Computer Group package 7.2.

Preparation of cell-free extracts and fractionated protein solutions. Two 2-litre flasks, each containing 1 litre of MM supplemented with 0.5% methanol or 0.1% succinate, were inoculated with 5 ml of a stationary-phase culture of an *M. organophilum* XX strain and incubated at 30 °C with shaking (225 r.p.m.). After the culture reached OD₆₀₀ 0.5, the culture was harvested by centrifugation (6000 r.p.m., 5 min). The cell pellets were resuspended in 10 mM Tris buffer (pH 7.9) and cell suspensions were pooled into a 30 ml centrifuge tube. Cells were centrifuged at 6000 r.p.m. for 5 min and the pellet was resuspended in the Tris buffer. The washings were repeated one more time. The pellet was resuspended thoroughly in 20 ml buffer B [12 mM HEPES/NaOH (pH 8.0), 4 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl]. The cell suspension was passed through a French pressure cell, twice at 15000 p.s.i. (103 MPa), to break the cells. The broken cell suspension was collected into a glass Corex tube (30 ml) and centrifuged for 30 min at 15000 g. The cell-free supernatant was carefully collected and was ready for use. Where appropriate, the extract was directly fractionated through a DNA-cellulose

(P. L. Biochemicals) column (0.5 cm × 15 cm) equilibrated with buffer B. The loaded column was washed with 10 ml buffer B. Bound proteins were eluted sequentially with buffer B (2 ml) containing increasing concentrations of salt (0.2, 0.4, 0.6, 0.8 and 0.9 M KCl).

Mobility-shift DNA-binding assays. These assays were performed based on the method of Chodosh (1989). Two DNA fragments containing the *mxoF* promoter region were used in the assay. A 353 bp DNA fragment was obtained by digesting plasmid pUS325-17 (Machlin, 1987) with *AvaI* and *HindIII*. A smaller DNA fragment (149 bp) encompassing nucleotides -94 to +43 of the promoter region (transcription starts at +1) was synthesized by PCR amplification of template pUS326-17. The 5' PCR primer is 5'-GAATTCGAGAAGATCGCCAA-3'. The 3' PCR primer is 5'-AAGCTTAGATAAACTCTGTC-3'. DNA fragments were end-labelled with [α -³²P]dATP and used as probes. A typical binding reaction contained the following in a volume of 15 µl: 12% glycerol, 150 pg DNA probe (approximately 5000 c.p.m.), 4.5 µg bovine serum albumin, 1 µg carrier DNA [either poly(dI-dC)·poly(dI-dC), or pUC118 plasmid], 30 µg or less crude protein extract or DNA-column-fractionated proteins and 20 ng competing unlabelled DNA fragment when appropriate. The binding reaction mixture was incubated for 15 min at 30 °C in a water bath. The reaction samples were loaded into wells of a 4% (w/v) polyacrylamide gel and electrophoresed at 100 V for 90 min at 4 °C using circulating low-ionic-strength buffer (6.7 mM Tris/HCl, pH 7.9; 3.3 mM sodium acetate; 1 mM EDTA, pH 7.9). The polyacrylamide gel was dried on a piece of Whatman paper and the dried filter was exposed to X-ray film overnight.

Deletion mutagenesis of the *mxoF* promoter region. The transcriptional fusion plasmid pHX200V-47 constructed previously (Xu *et al.*, 1993), in which *xylE* is under control of the promoter of *mxoF*, was used for deletional mutagenesis to identify sequences of the upstream region that are important for *mxoF* promoter activity and regulation. The digestion of the plasmid by restriction endonuclease, exonuclease III and S1 nuclease was performed as described by Sambrook *et al.* (1989). Plasmids with various lengths of deletion in the *mxoF* promoter region were mobilized into wild-type cells of *M. organophilum* XX, and transconjugants grown on MM plus methanol were assayed for expression of the *xylE* gene, as described previously (Xu *et al.*, 1993). The end-points of deletions were identified by double-stranded DNA sequencing, again as described previously (Xu *et al.*, 1993).

RESULTS

Previous work in this laboratory has indicated that a 5.5 kb *EcoRI* restriction fragment from a group VII cosmid contains sufficient sequence to complement *mxoQ* and *mxoE* mutant strains of *M. organophilum* XX (Machlin *et al.*, 1988). Further subcloning complementation analysis and transposon insertional mutagenesis have localized the *mxoQ* and *mxoE* genes within a 2.1 kb *BstEII* and *EcoRI* restriction fragment (Machlin *et al.*, 1988). Therefore, the 5.5 kb *EcoRI* restriction fragment was cloned into pBluescript II SK(+) and a 2.5 kb *BamHI*-*BglII* restriction fragment was removed from the recombinant plasmid, thus creating a plasmid with an insert size of 3.0 kb. A 2692 bp sequence of the insert was determined and is shown in Fig. 1.

DNA sequence analysis of this 2692 bp region revealed two open reading frames (ORFs) which appeared to

GGTGGCGCGCTCTAGAACTAGTGGATCTGGTCAACCGGACGTGACTTCAACGTCCCAAG 60	CCGAGACCGTGCCTGGTATGAGATCATCCGATCGTCTCCAGGGCTCCGCGCGGTGC 1980
CTGGCTCAATCCGAAGACTCTGGCAGGACTGGAACACCCCGACTCGGGCGACGCGTCCCG 120	A E T V L V I D D H P I V L Q G C R R V 38
CCCGAGCCCCCTCGCCCGGCAAGGTGGCCCGCGCGCTTCGAGAACTCCCGGACA 180	TGGAGGATGCCCGCATCGAGAGCGTGGCCGAGGCGACCGGCACGCTGCGCGGTACCGCC 2040
GGGGCAGCAATAAGAGATTCTCTAGTGCAGCTCGAGGCCCCAGCGGGCTAGCAAGAT 240	L E D A G I E S V A E A T G T V A G Y R 58
GGACTTTCCGGCGACCAATCGGCCACGCTCGCCGGCGGACGCGAAACGCTTCCATTGAG 300	TGTTCCACCGCTCGGCCCGAAGGTGTGATCTGCGACCTCACTTCCAGGGCAGCGGAT 2100
CGCTTAAACGTGACCGCACCCCGCAGCGCGCTTCCCGACCCGGGATCCCGCCCGCAA 360	L F H R L R P K V V I C D L T F Q G S G 78
CGGGGCTT <u>GAGGAGG</u> CGCGATGGGCCAGAGCGCGGATCAAGACGCTTCGAGGCGCG 420	TGGCGGGCTCGCCCTGATCCGCGCATCGCGCGCGTGGAGCCGGGCAACGCGGTGCTCG 2160
M G Q R P D H D A F E A A 13	L A G L A L I R R M R A V E P G T R V L 98
CGCCAGGACGGCCCCCGCCACCCGGGGCGCCCTGCCCTTCTGGCGCGGGCTGTCCACG 480	TGTTCTCGATGCAACGACCCCGTTCATCGTCTCGCGCGCCCTCGAAGCCGGGCGCTCG 2220
R Q E R P P A T R G A L P F W A G L S T 33	V F S M H N D P V I V S R A L E A G A L 118
CGGATCGCGCTGATCTCGTGTGCTCGCCATCGACCTGGTGGCGGCATCGCCACCTGC 540	GCTACGTGCTCAAGGACCAACCGCTCCCAAGAGCTGTTCAGGCTTTCAGGAGGTGCGCA 2280
R M R L I L V V L A I D L V A A I A T C 53	G Y V L K D H A S N E L F Q A F E Q V R 138
GGGTTCATCTGCTCAACCGCCGACAGCGGTGAAGGTGAGATCGCGCTCTCCCTGCGC 600	AGGGGAGGTCTTCCTCGACCGCGCGCTCGCCACCGAGGTGGCGATGCTCGCGCGCGACC 2340
G V I V L N A R Q A V K V E M R A S L A 73	K G E V F L D R R L A T E V A M L R A D 158
ACCGTCGAGCCCTCGTCTCGGACACGATCGCGCTGGCCCGGGCGCCACGCCCGAGAGC 660	CGCGCGCACCCCCGAATCCAGCTCACCCCGCGAGCAGCAGATCTGGCGCGCTCG 2400
T V E P L V S D T I R L A R A P S P E S 93	P R R T P E S Q L T P R E Q Q I L A R L 178
CTGCTCCACACCTCGATCTCGCTTCCAGACCTCGCGCCAGTCCGCGTCCGCTGTTC 720	CCAGGGCAAGTCTCGCGCGATCGCGCGGACCTCTCGGTGAGTACAGACCGTCA 2460
L L H T L D L R F Q T L R H V R V A V F 113	A Q G K S Y G A I A A D L S V S Y K T V 198
GACCGCGCGCGCAGCGGTGACCATCGACGAGCCAGGGCTGGCGCCCGCGCGCGGAG 780	CCAATGCTCTCGCAGATCGCGCAGAACTCGCGGTGCGCGCCCTGGCGGAGCTGATCC 2520
D A A G Q R V T I D E P G L R R R P R E 133	T N A C S Q M R Q K L G V R A L A E L I 218
GCGCGCAGCTGCTGCTGTCAGTGTGCGCGCGCGGTGGAGCAGCATCAGGTGCCGATC 840	ACGTGGCGTGAACCAAGCGCAGGGCAGGGTGAGACGTTCGCTTCGCTTTCGCTAGAGG 2580
A P H W F V Q L I A P P V E A Q H Q V P I 153	H V A V T Q A Q G R G E T S P P V A * 236
GTGGCGCGCGGCGACCATCGGCACCGCCCGCATCCACCGAGCGCGCGCAGCAGATC 900	GGTCGAGCGCTTTCCTTCCCTCTCGGGGAAGGTGGCGCGAAGCAGGAGCGCGCAC 2640
V A G A T I G T A R I T T E P G D E I 173	CGAAGTACCGGCATCGAGCGCGCGCGCTCCACCGCGCTTCCCGCTCG 2692
GAGGAGAGCTGGGACGACCGCTCTCCCTGTGCTGGCGAGCTGCTCTCAACGTGCG 960	
E E S W D D A V S L S L A S L L L N V A 193	
GTTCGGCGCGCTCTACCTCGCTTTCGGCGCGCTGCTGAAGCGCTGACCGCGCTGCGC 102C	
V L A A L Y L A F G R L L K P L H G L A 213	
GAAGGGCTGACCCAGCTGGAGCGGACGACTACACCGCGCGCTCGACCGCGGACCTCC 108C	
E G L T Q L E R H D Y T A R L D P P T S 233	
CGCGAGCTCGCGGTGATCACCGAGCGCTTCAACAGCGTGGCGGGCGCTGAGCGAGGCC 114C	
R E L A V I T E R F N S V A G A L S E A 253	
CGCTCGGCCAACCAACCGCTGAACCGCGAGCTCTGACCGCGCAGGACGACGAGCGCGC 120C	
R S A N N R L N R Q L L T A Q D D E R R 273	
CGCACCGCGCTGGAATTGCACGACGAGTTCGGGCGCTCGCTTGTGCGCTTCGAGGCAAT 126C	
R T A L E L H D E F G P C L F A L E A N 293	
GCGCGCTCGTGGCGCGCTCGGCCAGGCCCGGAGCGCGACCGCGCAAGCTCGCGGCC 132C	
A A S V A R L A Q A P E P D R A K L A A 313	
CGGGCGGGGACATCGCGGCATCGTGGGCGAGTGCAGACGCTCAACCGCGACCTGCTC 138C	
R A G D I A G I V G Q V Q T L N R D L L 333	
AACCGCTCGCGCCCCACCGCTGGGCGAGTCCCGCTCGCGCAATGCTTCGACCTGCTC 144C	
N R L R P H A L G E V P L P Q C L D L L 353	
CTGGCGGATTTTTCGCGCGCATCGGGCAGCGCTTCGAGGGCCATTTCGAGGATCTC 1500	
L R D F S R R H P G T R F E G H F E D L 373	
GGCGCGGCTACCGGACGCTCGTGCACCTACCGTGTTCGCTGCATCCAGGAGAGCATG 1560	
G R G Y G D V V D L T V F R C I Q E S M 393	
ACCAACCGCGTGCCTACCGGCGCGCGCCCGCATGTCGCGCGAGGCGCGGAGACGCG 1620	
T N A V R H G A A R H V R A E A G E T A 413	
CGGGCGGTGACGCTGAGCGTGGGGGACGCGGCGCGCTCGCCCGGACCGCGCAC 1680	
R G L T V S V R D P G G L A P D H R T 433	
GGCTTCGGCTCTCGCGATGCGGAGCGCGTGGAGGCCCTCGCGCGGACGCTTCAACCTT 1740	
G L G L S G M R E R V E A L G G S F T L 453	
GACAAATCGCGCGCGGAACCGTGTTCGCGATCAGATCCCGATCGATCCGAGCGGGG 1800	
D N A A P G T V V R I T I P I D P E R G 473	
GAGGACAGGCGCGCGGACCGCGCGCGCGCGCGCGCGCTCTCTCGAGACGAGCCC 1860	
E D Q A A G H P A P A A T P S L E T K P 493	
<u>mxrE</u> -->	
GCGACATGACTGCGCTTCGATGAAGACCCCGCGGACTCCCTCGCGCAAGGGCACCCCG 1920	
A T * 495	
M T A L A M K T P A D S L A K G T P 18	

Fig. 1. Nucleotide sequence of the 2692 bp DNA fragment and deduced amino acid sequences of the *mxrQ* and *mxrE* genes from *M. organophilum* XX. Putative ribosome-binding sites are underlined. Asterisks indicate the ends of the protein sequences.

correspond to the proposed *mxrQ* and *mxrE* positions and sizes (Fig. 1) (Machlin *et al.*, 1988). Both ORFs have putative ribosome-binding sites characteristic of *M. organophilum* XX genes. The *mxrQ* gene starts at nt 382 and ends at nt 1869, and would encode a 495 amino acid polypeptide with an estimated molecular mass of 54 kDa. The *mxrE* gene starts at nt 1866 and ends at 2576, encoding a polypeptide of 236 amino acids with an estimated molecular mass of 26 kDa. Gene bank searches revealed that *mxrQ* and *mxrE* belong to the histidine kinase and response regulator families, respectively.

MxrQ is a member of the histidine kinase superfamily

The C-terminal deduced amino acid sequence of *mxrQ* was found to be similar to MxaY [formerly MoxY (Lidstrom *et al.*, 1994)] of *Paracoccus denitrificans* (Harms *et al.*, 1993), and a number of other histidine kinases from this superfamily (Fig. 2). MxrQ and MxaY have the highest sequence similarity over entire amino acid sequences (29% identity over 420 amino acid overlap) (Fig. 2). As expected for a histidine kinase, MxrQ contained several residues that are especially conserved in its C-terminal portion. A conserved histidine precedes a conserved asparagine by about 100 residues (Stock *et al.*, 1989). A stretch of 15–45 amino acid residues separates this asparagine and a five-residue region designated DXGXG (where X residue is not conserved). Twenty to 50 residues away from DXGXG, there follows a GXG

Fig. 2. Amino acid sequence alignment of MxcQ with the entire MxaY sequence of *P. denitrificans* (Harms et al., 1993) and C-terminal parts of NarQ and NarX of *E. coli* (Nohn et al., 1989; Rabin & Stewart, 1992), DegS of *Bacillus subtilis* (Tanaka & Kawata, 1988; Henner et al., 1988), and UhpB of *Salmonella typhimurium* (Island et al., 1992). Numbers indicate MxcQ amino acid positions. Identical residues at a given position are marked by an asterisk at the bottom of the alignment. Residues of the same conserved amino acid groups are indicated by a dot. Putative transmembrane domains are underlined. Residues in bold face are highly conserved among histidine kinases.

conserved block. In the MxcQ protein sequence, H-280 is separated by 115 amino acids from conserved N-395, followed by a 27-residue spacing, and the conserved D-422XG-424XG-426. Interestingly, the GXG conserved regions in MxcQ (Fig. 2) and MxaY (Harms et al., 1993) are only 7 and 10 residues, respectively, away from the conserved DXGXG regions, a much shorter distance than in the majority of the histidine kinase family (Fig. 2) (Stock et al., 1989). Hydrophobicity analysis of MxcQ protein has located two potential transmembrane helices (positions 36–59 and 182–204 in Fig. 2), aligned similarly to the putative transmembrane helices of MxaY protein (Harms et al., 1993).

MxcE is a member of the response regulator family

The response regulator family is defined by a conserved domain of about 100 amino acids that are generally located near the N-terminus of these proteins (Stock et al., 1989). Based on sequence similarities between their C-terminal domains, these response regulators may be placed

into several subfamilies (Stock et al., 1989). The deduced amino acid sequence of *mxcE* was found to belong to a response regulator subfamily including MxaX [formerly MoxX (Lidstrom et al., 1994)] of *P. denitrificans* (Harms et al., 1993) (Fig. 3). MxcE has the highest sequence similarity to MxaX of *P. denitrificans* (35% identity in 217 amino acids overlap) (Fig. 3). As can be seen in Fig. 3, there are three amino acid residues universally conserved in the N-terminal domains of these response regulators: an aspartic acid residue corresponding to D-27 of MxcE, another aspartic acid residue corresponding to D-71 of MxcE, and a lysine residue corresponding to position K-123. The second aspartic acid position has been found to be the site of phosphorylation by histidine kinases of the two-component systems (Stock et al., 1989; Hess et al., 1988; Igo et al., 1989). The C-terminal sequence of MxcE was also found to align very well with the C-terminal domains of other proteins in this subfamily (Fig. 3), with the following characteristic patterns (Kahn & Ditta, 1991): (i) one block of similarity around the conserved Arg/Glu pair at R-170/E-171 of MxcE protein; (ii) a

Fig. 3. Amino acid sequence alignment of MxcE with MxaX of *P. denitrificans* (Harms et al., 1993), DegU of *B. subtilis* (Tanaka & Kawata, 1988; Henner et al., 1988), NarL of *E. coli* (Nohno et al., 1989), UhpA of *S. typhimurium* (Island et al., 1992), BvgA of *Bordetella pertussis* (Arico et al., 1989), RcsB of *E. coli* (Stout & Gottesman, 1990) and FixJ of *Azorhizobium caulinodans* (Kaminski & Elmerich, 1991). The site of phosphorylation is marked 'Pi'. Numbers indicate MxcE amino acid positions. Identical residues at a given position are marked by an asterisk at the bottom of the alignment. Residues of the same conserved amino acid groups are indicated by a dot. Residues in bold face are highly conserved among this subfamily of response regulators.

Fig. 4. Mobility-shift DNA-binding assays. (a) DNA-binding assay using a 353 bp *Ava*I and *Hind*III DNA fragment (150 pg DNA probe per well). Crude protein extract (lane 8) as well as DNA-column-fractionated proteins (eluted at 0.6 M KCl; named D fraction) (lanes 2–7) were added to the reaction mixture. These extracts were from *M. organophilum* XX grown in MM plus methanol. Lane 1, DNA probe control; lanes 2 and 3, 1.7 ng D fraction; lanes 4 and 5, 3.4 ng D fraction; lanes 6 and 7, 6.8 ng D fraction; lane 8, 10 µg crude protein extract; reactions in lanes 3, 5 and 7 each contained 20 ng unlabelled competitive DNA fragment. (b) DNA-binding assay using a 149 bp PCR-amplified DNA fragment (140 pg DNA probe per well). Lane 1, DNA probe control; lanes 2, 3 and 4 contain 6.8 ng, 13.6 ng and 27 ng, respectively, of D fraction; lane 5, 38 µg crude protein extract. The extracts were the same as above. (c) DNA-binding assay of mutant cellular extracts using a 149 bp PCR-amplified DNA fragment (140 pg DNA probe per well). Lane 1, 149 bp DNA probe control; lane 2, 25 µg of crude protein extract from mutant strain SM4 (an *mxcQ* mutant) grown with succinate; lane 3, 22 µg of crude protein extract from mutant strain SM49 (an *mxcE* mutant) grown with succinate; lane 4, 26 µg crude protein extract from wild-type strain grown with succinate; lane 5, 6.8 ng D fraction from the wild-type strain grown with methanol.

Nucleotide sequences and transcriptional start sites of *mxoF* genes from *M. organophilum* XX and *Methylo-*

bacterium extorquens AM1 have been identified (Machlin & Hanson, 1988; Anderson *et al.*, 1990). Not only are the two ORFs of the *mxaF* genes well conserved, but the two genes have identical -35 and -10 promoter hexamers as well as Shine-Dalgarno sequences (Machlin & Hanson, 1988; Anderson *et al.*, 1990). Due to the fact that *mxaF* was shown to be induced by methanol, an obvious point of regulation would be at the level of transcription. Mobility-shift DNA-binding assays (Fig. 4) were performed using a 353 bp *AvaI/HindIII* restriction fragment and a PCR-amplified 149 bp DNA fragment containing

Fig. 5. Deletional mutagenesis of the *mxaF* promoter fragment. (a) Nucleotide sequence of the *mxaF* promoter region (presented previously by Machlin & Hanson, 1988, and Xu *et al.*, 1993). Deletion end-points are individually marked 'mN', where 'm' means mutation and 'N' is the Arabic numeral designated for individual clones. The promoters (−35 and −10 hexamers) of *mxaF* (Machlin & Hanson, 1988) and *mxaW* genes [formerly *moxW* (Xu *et al.*, 1993; Lidstrom *et al.*, 1994)] are underlined. Dyad symmetry is indicated by converging arrows (>>> <<<). Nucleotides are numbered based on the definition of +1 for the *mxaF* transcription start site. Also shown are the positions of PCR primers used to synthesize the 149 bp DNA fragment used for DNA-binding assays. (b) Schematic representation of deletions and corresponding promoter activities of their transcriptional fusions with the promoterless *xylE* gene. *M. organophilum* XX strains harbouring various plasmids containing fusions were grown on MM plus methanol and catechol 2,3-dioxygenase activity was measured for each cell-free extract. Control, plasmid pHX200V-47 containing the wild-type promoter fragment (Xu *et al.*, 1993). Promoter activity is represented by specific activity (mU mg^{−1}) of catechol 2,3-dioxygenase. One milliunit corresponds to the formation of 1 nmol product min^{−1} at 30 °C. The background level of catechol 2,3-dioxygenase activity from a strain containing the promoterless *xylE* gene was not detectable. All assays were performed at least twice, and values agreed within +20%. ND, None detectable.

the *mxoF* promoter region from *M. organophilum* XX (the positions of the PCR primers are shown in Fig. 5a). A DNA band shift was observed when a crude extract or the protein fraction eluted by 0.6 M KCl/buffer B from the DNA-cellulose column was incubated with the promoter fragments (Fig. 4a, b). DNA-column fractionation was shown to greatly concentrate DNA-binding activity (data not shown). The retarded band decreased in intensity when an excess of competitive (unlabelled) DNA fragment was added, indicating the existence of DNA-binding protein(s) capable of binding specifically to the promoter region (Fig. 4a). On the other hand, the *mxoQ* mutant of *M. organophilum* XX grown in succinate produced no proteins that bound specifically to the *mxoF* promoter region (Fig. 4c), even though wild-type cells grown with succinate also produce protein(s) that bind to the promoter fragments (Fig. 4c). Succinate-grown cells have MDH with approximately 25% of the specific activity of methanol-grown cells (data not shown). A DNA band shift with reduced density was observed with the cell-free extract from the *mxoE* mutant (Fig. 4c).

Definition of the functional boundary of the *mxoF* upstream regulatory region

The difference in DNA-binding activities between extracts of *M. organophilum* XX wild-type strain and mutant strains implies a regulatory role for the *mxoF* upstream region. Therefore, deletions of the *mxoF* upstream region were constructed to confirm the importance of *mxoF* upstream sequence to regulation. Sequential deletions were made from the distal end of the *mxoF* upstream region on plasmid pHX200V-47 (Xu *et al.*, 1993) and the promoter activities of the mutant promoter regions were examined in *M. organophilum* XX based on the expression of the reporter gene *xylE*. As shown in Fig. 5(a, b), the first three deletions (m10, m9 and m1) from the distal (left) end had no apparent effects on *mxoF* promoter activity (*xylE* expression was almost the same as in the strain harbouring control plasmid pHX200V-47). This indicated that the deleted nucleotides (between -324 and -140 based on the transcription start at +1) were not important in regulating *xylE* expression. Deletion m2 caused a 59% reduction in promoter activity, although the -35 hexamer of the promoter is still 76 bp away. The next three deletions (m5, m11 and m8) completely abolished the promoter activity. These results indicated that nucleotides between -103 and -62 were essential for *mxoF* promoter activity. The sequence immediately upstream of -103 may be involved in the regulation of *mxoF*, but it is not essential.

DISCUSSION

In this report, we have described the nucleotide sequence of the *mxoQ* and *mxoE* genes required for the synthesis of methanol dehydrogenase in *M. organophilum* XX. Through database searches, the deduced amino acid sequences of MxcQ and MxcE polypeptides were shown to show similarity with the histidine kinase and response

regulator families, respectively. Especially significant is the finding that MxcQ and MxcE proteins have the highest similarity with another pair of two-component regulators, MxaY and MxaX, respectively, of the methylophilic bacterium *P. denitrificans* (Figs 2 and 3; Harms *et al.*, 1993). The amino acid residues that are conserved among histidine kinases and response regulators (Stock *et al.*, 1989) are also found in the MxcQ and MxcE proteins (Figs 2 and 3). These observations imply that the *mxoQ* and *mxoE* genes of *M. organophilum* XX are equivalent to the *mxoY* and *mxoX* genes of *P. denitrificans*, although their locations relative to the regulated genes (*mxoF*) are different. *mxoY* and *mxoX* are linked to *mxoF* on the chromosome of *P. denitrificans* (Harms *et al.*, 1998), while *mxoQ* and *mxoE* are located at least 40 kb away from the gene cluster containing *mxoF* (Machlin *et al.*, 1988). As for *mxoY* and *mxoX*, there is no intergenic sequence between the *mxoQ* and *mxoE* coding sequences, implying co-transcription of these two genes (Fig. 1).

Mutant strains of *M. organophilum* XX with defects in *mxoQ* and *mxoE* have previously been isolated (Machlin *et al.*, 1988). These mutants (formerly designated VII-A, VII-B mutants) cannot grow on methanol, but retain the ability to grow on methylamine and succinate (Machlin *et al.*, 1988). During growth on methylamine, *mxoQ* and *mxoE* mutants do not synthesize proteins that cross-react with anti-MDH antisera (Machlin *et al.*, 1988). Furthermore, no reporter gene transcription was detected in these mutants when they harboured plasmids containing *mxoFp::xylE* transcriptional fusions (Xu *et al.*, 1993). The discovery in this study that *mxoQ* and *mxoE* genes probably encode a histidine kinase and a response regulator, respectively, not only reaffirms the conclusion that *mxoQ* and *mxoE* genes are required for the synthesis of MDH (Machlin *et al.*, 1988; Xu *et al.*, 1993; Lidstrom *et al.*, 1994) but also indicates the possible mechanism of regulation involved.

It is reasonable to propose that, like other pairs of two-component systems (Nohn *et al.*, 1989; Stock *et al.*, 1989), *mxoQ* and *mxoE* are constitutively produced by *M. organophilum* XX cells. After sensing the signal (whose identity is not yet known), MxcQ protein may auto-phosphorylate itself, thus activating its kinase activity, presumably located at its C-terminus. Then MxcQ phosphorylates the MxcE protein present in the cytoplasm. Activated MxcE protein may bind to the promoter region of the *mxoF* gene (possibly other genes), activating its transcription. DNA-binding results indicated that the *mxoQ* mutant does not produce protein(s) that bind to the *mxoF* promoter region, consistent with the role of a histidine kinase. Less clear is the result of DNA binding by extracts of the *mxoE* mutant; the cell-free extract still contained DNA-binding activity, although at a reduced level. This can be explained by the possibility of a mutation in MxcE protein that renders it incapable of activating *mxoF* transcription but does not prevent its binding to the target site upstream of *mxoF*. Alternatively, MxcE may activate the transcription of yet another regulatory protein which directly activates the *mxoF* gene. The presence of a putative helix-turn-helix domain

in the MxcE protein supports its function as a DNA-binding protein, just like most other response regulators (Stock *et al.*, 1989).

In vivo analyses of deletion mutant plasmids have indicated that nucleotides upstream of -140 (relative to the transcription start of *mxoF*) had no effect on the function of *mxoF* promoter fused to the *xylE* gene and nucleotides between -140 and -103 only slightly affected the activity of the *mxoF* promoter. In contrast, nucleotides between -103 and -62 were absolutely essential for the expression of *xylE*, implying a positive regulatory mechanism for control of the *mxoF* promoter. Interestingly, a dyad symmetry was found to span nucleotides -91 and -60 (Fig. 5a), providing a potential binding site for a positive regulatory factor.

de Vries *et al.* (1990) proposed that a GC-rich consensus sequence appearing upstream of several *P. denitrificans* genes is responsible for regulating gene expression in that bacterium. Support for this hypothesis was found in *M. organophilum* XX because a sequence with partial similarity to this consensus was located more than 300 bp upstream of the transcriptional start site of *mxoF* from strain XX (de Vries *et al.*, 1990). However, the results of deletion analysis clearly indicated that nucleotides upstream of position -140 are not required for normal transcription and regulation of *mxoF* in *M. organophilum* XX.

ACKNOWLEDGEMENTS

This work was funded by a grant from the US Department of Energy (DOE-F602-88ER 13682). We would like to thank anonymous reviewers for their comments to improve the manuscript.

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Received 15 February 1995; revised 16 May 1995; accepted 7 June 1995.