Identification of methanol-regulated promoter sequences from the facultative methylotrophic bacterium *Methylobacterium organophilum* XX

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(Received 21 August 1992; revised 18 November 1992; accepted 8 December 1992)

A promoter-probe vector (pHX200) was constructed using the broad-host-range cosmid pLA2917 and a promoterless *xylE* gene of *Pseudomonas* as the reporter gene. Insertion of the cloned promoter fragment of the methanol dehydrogenase large subunit gene *moxF* (methanol oxidation) in front of the *xylE* gene in pHX200V-47 resulted in high-level expression of the *xylE* gene product – catechol 2,3-dioxygenase – in *Methylobacterium organophilum* XX. The specific activity of the enzyme was four times higher in methanol-grown *M. organophilum* XX culture than in succinate-grown culture. Interestingly, the insertion of the same fragment in the opposite orientation in front of the *xylE* gene (pHX200V-74) also led to elevated catechol 2,3-dioxygenase activity. This promoter activity was also methanol regulated. A total of 21 methanol-regulated promoter clones were identified that originate from three gene clusters (groups V, VI and VII) on the *M. organophilum* XX chromosome involved in methanol oxidation. Vector pHX200 and its derivatives were successfully mobilized into cells of three phylogenetically diverse methylotrophic bacteria: *Methylophilus methylotrophus* AS1, *Methylobacterium extorquens* AM1 and *Methylobacterium* sp. DM4. The reporter gene (*xylE*) was functionally expressed in all three bacteria with the aid of a proper promoter. Transcriptional fusions of methanol-regulated promoters with the *xylE* gene were mobilized into Mox⁻ mutants of *M. organophilum* XX and *M. extorquens* AM1 to study the roles of methanol oxidation genes, especially regulatory genes. It appeared that vector pHX200 is an efficient promoter probe with wide host-range and an excellent tool for studies of structure and function of promoters/regulators.

Introduction

Methylotrophic bacteria use one-carbon compounds more reduced than carbon dioxide as carbon and energy source. Carbon assimilation occurs at the oxidation level of formaldehyde (Anthony, 1982). *Methylobacterium organophilum* XX is a pink-pigmented facultative methylotrophic bacterium that utilizes methanol, methylamine, sugars, fatty acids and complex nutrients for growth (Patt *et al.*, 1976). It is a type II methylotroph that utilizes the serine pathway for formaldehyde assimilation. Type I methylotrophs utilize the hexulose monophosphate pathway for the same purpose (Anthony, 1982). 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). It is a periplasmic enzyme and contains the prosthetic group pyrroloquinoline quinone (PQQ). This enzyme has been found in all Gram-negative methylotrophic bacteria examined. The MDH enzymes are tetramers of two types of subunits in an $\alpha_2\beta_2$ conformation (Anthony, 1986; Cox *et al.*, 1992; Nunn *et al.*, 1989). The large subunit (α subunit) has a molecular mass of 56 to 76 kDa, while the small subunit (β subunit) has a molecular mass of 8 to 10 kDa (Anthony, 1982, 1986; Cox *et al.*, 1992; Nunn *et al.*, 1989). The MDH large subunit structural gene (*moxF*) and its upstream region (492 bp) from *M. organophilum* XX have been cloned and sequenced, and its transcriptional start site and promoter have been identified (Machlin & Hanson, 1988). Genetic studies of MDH synthesis in *M. organophilum* XX indicated (Allen & Hanson, 1985; Machlin *et al.*, 1987, 1988; Bastien *et al.*, 1989) that at

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Abbreviations: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; S-D, Shine-Dalgarno.

least seven separate chromosomal regions (groups I to VII) encode at least 15 genes that are required for methanol oxidation and formaldehyde assimilation, and that a total of five genes, including the MDH structural genes *moxF* and *moxI*, map within group V.

Despite identification of gene clusters required for methanol oxidation, the mechanisms of regulation of *moxF* and other related genes in methylotrophic bacteria remain unclear. To study the structure and function of methanol-regulated promoters, the availability of a promoter-probe vector applicable in methylotrophs is essential. Previous attempts to use *phoA* and *lacZ* as reporter genes in *M. organophilum* XX have been unsuccessful (R. S. Hanson, unpublished data). Recently, the catechol 2,3-dioxygenase gene (*xylE*) from *Pseudomonas putida* has been used successfully as the reporter gene in a number of phylogenetically diverse micro-organisms in which other reporter genes were poorly expressed (Zukowski *et al.*, 1983; Ingram *et al.*, 1989; Ray *et al.*, 1985, 1988; Winstanley *et al.*, 1989; Hahn *et al.*, 1991). Therefore, the *xylE* gene was selected as a candidate for a reporter gene for constructing a promoter-probe vector mobilizable into *M. organophilum* XX and other methylotrophs.

This study was undertaken to construct a promoter-probe vector and use it to identify methanol-regulated promoter sequences from *M. organophilum* XX. Moreover, expression of transcriptional fusion constructs was examined in *Mox*⁻ mutants of both *M. organophilum* XX and *Methylobacterium extorquens* AM1.

Methods

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium at 37 °C. *Methylophilus methylotrophus* AS1 was grown in CJ medium (Windass *et al.*, 1980) plus methanol at 37 °C. *Methylobacterium organophilum* XX, *Methylobacterium extorquens* AM1 and *Methylobacterium* sp. DM4 were grown in MacLennan minimal medium (MM) (MacLennan *et al.*, 1971) containing 0.5% (v/v) methanol or 0.1% (w/v) succinate at 30 °C. When required, antibiotics were added to final concentrations of 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; 1.5 µg ml⁻¹ of Tc for *Methylophilus methylotrophus* AS1; and 4 µg ml⁻¹ of Tc for *Methylobacterium extorquens* AM1 and *Methylobacterium* sp. DM4.

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

PCR amplification of the *xylE* gene. Oligonucleotide primers (XYLE primer1 5'-CAAGATCTGAATTCAGGAGGTGACGTCATG-3'; XYLE primer2 5'-CCCTGCAGGACCATCAGGTC-3') were designed based on the *xylE* nucleotide sequence (Zukowski *et al.*, 1983)

and synthesized by American Synthesis Inc. A modified promoterless *xylE* gene was synthesized by PCR based on the protocol of Perkin Elmer/Cetus Co.

Bacterial matings. Donor *E. coli* cells (containing mobilizable plasmids) were mated with *Methylobacterium organophilum* XX (wild-type or mutants), *M. extorquens* AM1, *Methylobacterium* sp. DM4 and *Methylophilus methylotrophus* AS1 for mobilization of plasmids as described previously (Allen & Hanson, 1985), with modifications. Matings involving *Methylophilus methylotrophus* AS1 as recipient were performed on a mixed medium of CJ and Antibiotic medium 3 (Difco) (ratio = 1/1). The others were performed on Antibiotic medium 3. Selection of transconjugants was achieved by replica plating onto MM-methanol plates (wild-type) or MM-methanol-succinate plates (mutants) with appropriate concentrations of tetracycline. CJ minimal medium replaced MM when *Methylophilus methylotrophus* AS1 transconjugants were selected. Plates were incubated for 3 to 5 d at 30 °C for most species except *Methylophilus methylotrophus* AS1, which was grown at 37 °C. Colonies of transconjugants were streaked on fresh plates of the same medium for purification.

Catechol 2,3-dioxygenase activity. Assays for the enzyme were performed as described by Zukowski *et al.* (1983) and Kataeva & Golovleva (1990), with slight modifications. Functional expression of the *xylE* gene was examined by spraying filters (Whatman filter paper no. 3) onto which colonies were replicated with a fresh aqueous solution of 0.1 M-catechol. Catechol 2,3-dioxygenase transformed catechol into a yellow product, 2-hydroxymuconic semialdehyde. Colonies of cells that expressed *xylE* became yellow or produced the diffusible yellow product. For quantitative assays, the enzyme reaction was conducted in a cuvette (light-path 1 cm) in a total volume of 1 ml containing: 0.8 ml 50 mM-Tris buffer (pH 7.5); 0.1 ml 10 mM-catechol dissolved in the Tris buffer; 0.1 ml cell-free protein extract or dilutions. Cell-free extracts were prepared as described by Machlin *et al.* (1987). The rate of reduction was taken as two times the change in absorbance at 375 nm between 15 and 45 s after mixing the components of the reaction. One milliunit corresponds to the formation of 1 nmol 2-hydroxymuconic semialdehyde per min at 30 °C. Protein concentrations were measured by the method of Bradford (1976), using bovine serum albumin as standard.

Primer extension. Total RNA was isolated from *M. organophilum* XX by the method of Zhu & Kaplan (1985). Hybridization of purified RNA (20 µg) with 4 × 10⁵ c.p.m. of radiolabelled oligonucleotide primer and primer extension were performed as described by Kingston (1989).

Identification of methanol-regulated promoters. Cosmids pS1033, pS150 and pS23 with inserts of *M. organophilum* XX chromosomal DNA including group V, VI and VII complementation clusters involved in methanol oxidation (Machlin *et al.*, 1987) were partially digested with *Sau3A* and ligated into the *Bam*HI site of pHX200. *E. coli* JM109 competent cells were transformed by the ligation mixture and tetracycline-resistant transformants were mated, by replica plating, with wild-type *M. organophilum* XX cells. Transconjugants grown on MM-methanol-Tc plates were sprayed with catechol to identify clones in which inserts promoted the expression of *xylE*. In order to exclude promoter sequences derived from the pLA2917 vector, each recombinant plasmid was digested with *Pst*I and fractionated on 0.9% agarose gel. Southern blots were prepared and hybridized with the radiolabelled plasmid vector pLA2917 based on protocols of Schleicher & Schuell, Inc. Those inserts that hybridized to the vector were excluded from further studies. Insert sizes were estimated after agarose gel electrophoresis.

Sequencing of promoter inserts. Two oligonucleotide primers were designed for sequencing of promoter inserts from both upstream and

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> JM109	<i>recA1 endA1gyr A96 thi hsdR17(r_K⁻ m_K⁺) supE44 Δ(lac-proAB) relA1 [F' traD36 proAB lacI^qZΔM15]</i>	Promega
<i>Methylobacterium organophilum</i> XX		
Wild-type (ATCC 27886)	Mox ⁺	This laboratory
SM37	Mox ⁻ , <i>moxB</i> /V-A1 mutant	
SM29	Mox ⁻ , <i>moxA</i> /V-B1 mutant	
SM35	Mox ⁻ , <i>moxI</i> /V-B2 mutant	
SM8 and SM28	Mox ⁻ , <i>moxF</i> /V-C mutants	
SM10 and SM19	Mox ⁻ , <i>moxM</i> /VI-A mutants	
SM2	Mox ⁻ , <i>moxN</i> /VI-B mutant	
SM48 and SM3	Mox ⁻ , <i>moxD</i> /VI-C mutants	
PT34 and PT47	Mox ⁻ , <i>moxO</i> /VI-D mutants	
SM4, SM18 and SM50	Mox ⁻ , <i>moxQ</i> /VII-A mutants	
SM16 and SM49	Mox ⁻ , <i>moxE</i> /VII-B mutants	
<i>Methylobacterium extorquens</i> AM1		
Wild-type	Mox ⁺	P. Goodwin, Wellcome Trust, London, UK
UV21	Mox ⁻ , <i>moxK</i> mutant	Nunn & Lidstrom (1986a)
M15A	Mox ⁻ , <i>moxL</i> mutant	
UV4 and UV25	Mox ⁻ , <i>moxB</i> mutants	
UV45 and UV40	Mox ⁻ , <i>moxC</i> mutants	
UV27	Mox ⁻ , <i>moxD</i> mutant	
AA31 and AA32	Mox ⁻ , <i>moxE</i> mutants	
UV26	Mox ⁻ , <i>moxF</i> mutant	
UV10 and UV24	Mox ⁻ , <i>moxG</i> mutants	
UV48	Mox ⁻ , <i>moxH</i> mutant	
<i>Methylobacterium</i> sp. DM4	Mox ⁺	T. Leisinger, ETH Zürich, Switzerland
<i>Methylophilus methylotrophus</i> AS1	Mox ⁺	ICI, UK
Plasmids		
pLA2917	Km ^r Tc ^r	Allen & Hanson (1985)
pRK2013	Km ^r , self-conjugative	Figurski & Helinski (1979)
pS1033	Contains group V large fragment	This laboratory
pS150	Contains group VI large fragment	
pS23	Contains group VII large fragment	
pTG402	Cm ^r Ap ^r <i>xylE</i>	Zukowski <i>et al.</i> (1983)
pSP72	Ap ^r	Promega
pHX151	Tc ^r <i>xylE</i> (Xyl ⁺)	This study
pHX200	Tc ^r , promoterless <i>xylE</i> (Xyl ⁻)	
pHX200V-47	Tc ^r , <i>moxFp::xylE</i>	
pHX200V-74	Tc ^r , <i>moxWp::xylE</i>	
pHX200VII-2	Tc ^r , <i>moxUp::xylE</i>	
pHX200VI-27	TC ^r , containing a methanol-regulated promoter VI-27	

downstream halves of the interrupted polylinker. The sequence of the upstream primer is 5'-TGCCTGCAGGTCGAC-3' and the sequence of the downstream primer is 5'-AGATCTGATATCATC-3'. Double-stranded plasmid DNAs were directly used for sequencing based on protocols provided with the Sequenase Version 2.0 Sequencing Kit by US Biochemical.

Nucleotide sequence access number. The nucleotide sequence (500 bp) of the *moxF* upstream region was determined by Machlin & Hanson (1988) and has been deposited in GenBank (accession number LO 7547).

Results

Construction of promoter-probe vector

A promoterless *xylE* gene was synthesized by PCR amplification from plasmid pTG402. The original *xylE* gene was expressed in *Methylobacterium organophilum* XX but not at a high enough level to be used as a reporter gene in the pink-coloured cells (H. H. Xu,

Fig. 1. Construction of promoter-probe vector pHX200. (a) DNA manipulation scheme; (b) S-D sequence comparison of the original and modified *xylE* genes. Abbreviations: P, *Pst*I; B, *Bgl*II; H, *Hind*III; c, cos site; Km, kanamycin; Tc, tetracycline; ---, *moxF* open reading frame.

unpublished data). The most probable reason for poor expression was low efficiency of ribosomal binding on the *xylE* mRNA in *M. organophilum* XX cells. Therefore, the S-D (Shine-Dalgarno) sequence of the *xylE* gene was modified during PCR amplification of the gene to resemble the S-D sequence of the *moxF* gene (Fig. 1b). As shown in Fig. 1(a), plasmid pLA2917 was digested with *Bgl*II and *Pst*I, sites for which reside within the kanamycin-resistance gene. The large fragment was ligated with the *Bgl*II- and *Pst*I-digested promoterless *xylE* gene fragment, producing construct pHX151 with a defective kanamycin-resistance gene. Plasmid pHX151, when mobilized into *M. organophilum* XX, expressed *xylE* efficiently due to the intact kanamycin promoter. The *Hind*III-*Bgl*II fragment on pHX151 containing the kanamycin promoter was replaced by the polylinker fragment from pSP72, producing the vector pHX200 whose *xylE* was not expressed in *M. organophilum* XX

Fig. 2. *moxF* promoter activity during growth of *M. organophilum* XX. *M. organophilum* XX cells were grown in methanol minimal medium plus tetracycline. The bacterium contained plasmid construct pHX200V-47, in which *xylE* is under control of the *moxF* promoter. The *moxF* promoter activity was indicated by *xylE* reporter gene expression. ○, Catechol 2,3-dioxygenase specific activity; ●, bacterial growth. Experiments were replicated at least twice; each data point is the mean of three replicate assays from a single experiment.

unless an active promoter was inserted within the polylinker. The polylinker contains the following unique restriction sites: *Hind*III, *Xba*I, *Bam*HI, *Kpn*I, *Sac*I and *Bgl*II.

Cloning of *moxF* promoter in front of *xylE*

A 448 bp *Kpn*I fragment containing the promoter region of the *moxF* gene was excised and inserted into the *Kpn*I site of the vector pHX200. The recombinant plasmids were mobilized into *M. organophilum* XX cells. Six transconjugant clones of *M. organophilum* XX produced a yellow colour after spraying with catechol solution. One of the clones was selected for sequence confirmation. The recombinant plasmid (designated pHX200V-47) was isolated from the corresponding *E. coli* JM109 strain and was sequenced using Sequenase Version 2.0. This plasmid contained an insert with a sequence identical to that of the *moxF* upstream promoter region. This fragment was inserted in front of *xylE* in the same orientation as that of the *moxF* promoter sequence.

The *M. organophilum* XX clone containing recombinant plasmid pHX200V-47 was grown in liquid MM-methanol-Tc medium at 30 °C, and cell growth and *xylE* activity were monitored (Fig. 2). The specific activity of catechol 2,3-dioxygenase (the *xylE* gene product) increased at a similar rate as cell density from lag phase to OD₆₀₀ 0.25 and then remained relatively

Fig. 3. Identification of the transcription start site of *moxW*. Autoradiography of primer extension experiment. G, A, C and T represent lanes loaded with standard sequencing reactions; PE marks the lane loaded with primer extension. The arrow points to the DNA band that was extended from the primer.

constant between OD₆₀₀ values of 0.2 and 0.7. Therefore, the assays for catechol 2,3-dioxygenase activity were performed using cell-free extracts obtained from cultures at OD₆₀₀ 0.5.

Discovery of an upstream divergently transcribed gene

Computer analysis of the upstream nucleotide sequence of *moxF* revealed one open reading frame that may be transcribed divergently from the *moxF* gene. This gave rise to the hypothesis that a divergently transcribed gene upstream of *moxF* exists which might be inducible by methanol and involved in methanol oxidation. If this were the case, we should be able to find a clone among the above six transconjugants which contains a plasmid with the 448 KpnI fragment inserted in the opposite

orientation to that in pHX200V-47. To test this hypothesis, five additional positive promoter plasmids identified above that promoted *xylE* expression in *M. organophilum* XX were sequenced. Three of them contained 448 bp inserts with the same insertion orientation as that in pHX200V-47 (see above); two others contained the same inserts in the opposite orientation (designated pHX200V-74). This indicated that the putative upstream divergently transcribed gene has an active promoter. This promoter also was shown to be regulated by methanol (see below). This upstream divergently transcribed gene is named *moxW*.

Transcriptional start site of the *moxW* gene

The *M. organophilum* XX clone containing pHX200V-74 was grown in MM-methanol-Tc medium to allow transcription of the *xylE* gene controlled by the *moxW* promoter. The oligonucleotide primer used in primer extension reactions was the same as the downstream primer used for sequencing. Primer extension experiments (Fig. 3) identified the site of transcriptional start for the *moxW* gene (W + 1) to be 143 bp upstream of the *moxF* transcriptional start site (Fig. 4).

Identification of methanol-regulated promoter clones

To further test the ability of the vector to 'capture' unknown promoter sequences from *M. organophilum* XX DNA and also provide material for future studies of promoters and regulators in this organism, three large DNA fragments (25 to 30 kb) containing three gene clusters involved in methanol oxidation were used to identify methanol-regulated promoter sequences. Cosmids containing the fragments were digested with *Sau3A* and ligated with *Bam*HI-cleaved pHX200. *E. coli* JM109 competent cells were transformed with the ligation mixture. Transformants were mated with wild-type *M. organophilum* XX cells. Transconjugants were screened for positive promoter clones in which inserted fragments promoted expression of the *xylE* gene. Thirty-two positive promoter clones were identified. After further screening, only 19 clones were determined to be methanol-regulated promoters. The other 13 were excluded either because the promoter sequences were derived from the cosmid vector or because they were also strongly expressed in succinate minimal medium. Qualitative results showed that *M. organophilum* XX clones containing these 19 plasmids produced a high level of catechol 2,3-dioxygenase when they were grown on a medium containing methanol, while they exhibited much lower or no catechol 2,3-dioxygenase activity when they were grown on succinate. Besides the promoters of *moxF* and *moxW* (whose sequence has been published), six of

Fig. 4. Transcriptional start site of *moxW* and its surrounding nucleotide sequence. Transcriptional start sites (designated as W + 1 and F + 1, respectively) and transcription direction of the *moxW* and *moxF* genes are indicated by arrows. Open reading frames are also indicated by arrows. Shine–Dalgarno (S–D) sequences, and –10 and –35 hexamers of the *moxW* and *moxF* promoters, are underlined. Repeated septanucleotides are indicated by bold letters. The dyad symmetry is indicated by >>>> <<<<.

19 promoter fragments were sequenced (H. H. Xu and others, unpublished data) and they represent six different sequences. For the rest of the promoter fragments, it is possible that some are duplicates within each group (e.g. group V, VI or VII).

Induction of promoter activity by methanol

Four promoter clones were examined for quantitative expression of the *xylE* gene (i.e. promoter activity) in the presence and absence of methanol. Clones of *M. organophilum* XX containing pHX200V-47, pHX200V-74, pHX200VI-27 or pHX200VII-2 were grown in liquid MM-methanol-Tc and MM-succinate-Tc media. Cells were harvested at OD₆₀₀ 0.5 and cell-free extracts were made. The catechol 2,3-dioxygenase activity of each clone was measured under each growth condition (Fig. 5). The results indicate that under control of these four promoters, the *xylE* gene was expressed at higher levels in methanol-grown cells than in succinate-grown cells. The *moxF* promoter in the pHX200V-47 construct produced the highest level of methanol-induced *xylE* expression. This was not surprising since MDH (*moxF*

encodes the large subunit of MDH) comprised 15% of the total protein of *M. organophilum* XX during growth on methanol (P. E. Tam & R. S. Hanson, unpublished data). The *moxF* promoter gave a lower but significant amount of *xylE* gene expression during growth on succinate. This is consistent with previous results which have shown that methanol dehydrogenase is synthesized at a relatively high background level, indicating loose regulatory control (O'Connor & Hanson, 1977). In contrast, *xylE* expression controlled by the promoter of *moxW* in the pHX200V-74 construct was shown to be under strict regulation. The *xylE* specific activity was 23 mU mg⁻¹ in presence of methanol but was zero (or below detection level) in absence of methanol.

Host range of vector pHX151, pHX200 and pHX200V-47

Vector pHX200, its parent pHX151 and one promoter-containing derivative (pHX200V-47) were mobilized into three other phylogenetically diverse methylotrophic bacteria (*Methylobacterium extorquens* AM1, *Methylobacterium* sp. DM4 and *Methylophilus methylotrophus*

Fig. 5. Comparison of promoter activities under different growth conditions. *M. organophilum* XX clones containing one of the four plasmids (pHX200V-47, pHX200VI-27, pHX200VII-2 and pHX200V-74) carrying various promoter clones were grown in either methanol or succinate minimal media. Experiments were replicated at least twice; each data point is the mean of three replicate assays from a single experiment.

AS1) (Tsuji *et al.*, 1990). The results showed that all four clones of methylotrophic bacteria containing pHX151 (in which the kanamycin promoter controls *xylE* expression) became yellow-coloured after spraying with a catechol solution. This indicates that *xylE* (at least the modified *xylE*) is an efficient reporter gene in these bacterial systems. Introduction of pHX200 vector did not lead to expression of *xylE* in three of the four methylotrophs (*M. organophilum* XX, *M. extorquens* AM1 and *Methylobacterium* sp DM4). This indicates that the vector is useful as promoter probe vector in these three bacterial systems. This observation was confirmed by the high-level expression of *xylE* by these cells once a methanol-regulated promoter (the *moxF* promoter) was inserted into pHX200. The vector cannot be used in *Methylophilus methylotrophus* AS1 without further modifications, because *xylE* of pHX200 was expressed in this bacterium.

Two classes of *Mox*⁻ mutants of *M. organophilum* XX and *M. extorquens* AM1

For convenience of discussion, the putative gene controlled by a newly identified methanol regulated promoter VII-2 (carried by plasmid pHX200VII-2) is tentatively named *moxU*. Transcription fusion plasmids pHX200V-47 (*moxFp::xylE*), pHX200V-74 (*moxWp::xylE*) and pHX200VII-2 (*moxUp::xylE*) were individually mobilized into each of 17 *Mox*⁻ mutants of *M. organophilum* XX. These 17 mutants represent 10 loci involved in methanol oxidation. Results with pHX200V-47 showed that, as expected, mutations in the structural

Table 2. Expression of the *xylE* gene fused to different promoter regions in *Mox*⁻ mutants of *M. organophilum* XX

Mutant	Locus	Transcriptional fusion		
		<i>moxFp::xylE</i>	<i>moxWp::xylE</i>	<i>moxUp::xylE</i>
SM37	<i>moxB</i>	—	+	+
SM4	<i>moxQ</i>	—	+	+
SM18	<i>moxQ</i>	—	+	+
SM50	<i>moxQ</i>	—	+	+
SM16	<i>moxE</i>	—	+	+
SM49	<i>moxE</i>	—	+	+
SM10	<i>moxM</i>	—	—	—
SM19	<i>moxM</i>	—	—	—
SM2	<i>moxN</i>	—	—	—
SM48	<i>moxD</i>	—	—	—
SM3	<i>moxD</i>	—	—	—
SM29	<i>moxA</i>	+	+	—
SM35	<i>moxI</i>	+	+	—
SM8	<i>moxF</i>	+	+	—
SM28	<i>moxF</i>	+	+	—
PT34	<i>moxO</i>	+	+	—
PT47	<i>moxO</i>	+	+	—

Table 3. Expression of the *xylE* gene fused to different promoters in *Mox*⁻ mutants of *M. extorquens* AM1

Mutant	Locus	Transcriptional fusion	
		<i>moxFp::xylE</i>	<i>moxWp::xylE</i>
UV4	<i>moxB</i>	—	+
UV25	<i>moxB</i>	—	+
AA31	<i>moxE</i>	—	+
AA32	<i>moxE</i>	—	+
UV27	<i>moxD</i>	—	—
UV21	<i>moxK</i>	+	+
M15A	<i>moxL</i>	+	+
UV40	<i>moxC</i>	+	+
UV45	<i>moxC</i>	+	+
UV26	<i>moxF</i>	+	+
UV10	<i>moxG</i>	+	+
UV24	<i>moxG</i>	+	+
UV48	<i>moxH</i>	+	+

genes of MDH large subunit (*moxF*) and small subunit (*moxI*) did not affect the expression of the *xylE* gene (Table 2). On the other hand, mutations in the *moxQ* and *moxE* genes previously implicated as regulatory genes (Nunn & Lidstrom, 1986b; Lidstrom, 1990) were confirmed by this assay to be involved in regulation of *moxF* transcription. Mutations on loci *moxA* and *moxO* did not affect *moxF* expression. But the opposite results were observed in mutants defective in loci *moxB*, *moxM*, *moxN* and *moxD*. In comparison, *moxW* promoter activity was affected only by mutations in the *moxM*, *moxN* and *moxD* genes (Table 2). Furthermore, the activity of the *moxU* promoter carried by pHX200VII-2

was inhibited by most mutations except those in the *moxB*, *moxQ* and *moxE* genes (Table 2).

Transcriptional fusion plasmids pHX200V-47 (*moxFp::xylE*) and pHX200V-74 (*moxWp::xylE*) were mobilized into 13 *Mox*⁻ mutants of *M. extorquens* AM1. Mutations in *moxK*, *moxL*, *moxC*, *moxF*, *moxG* and *moxH* did not influence the activity of either the *moxF* promoter or the *moxW* promoter (Table 3). Mutations in two regulatory loci (*moxB* and *moxE*) abolished activity of the *moxF* promoter but not activity of the *moxW* promoter (Table 3). Homogenous loci from *M. extorquens* AM1 and from *M. organophilum* XX had identical effects on both *moxFp::xylE* and *moxWp::xylE* fusions (Tables 2 and 3).

Discussion

A number of vectors employing *xylE* as the reporter gene have been developed in systems where well-established reporter genes such as *lacZ* were not very effective (Zukowski *et al.*, 1983; King & Chater, 1986; Ray *et al.*, 1988, 1985; Winstanley *et al.*, 1989). In contrast, very little has been reported on the application of reporter genes in methylotrophs. Attempts to use *lacZ* and *phoA* as reporter genes in *M. organophilum* XX were unsuccessful (R. S. Hanson, unpublished data). Recently, Morris & Lidstrom (1992) utilized the *lacZ* gene as the reporter gene and studied the promoter activity of the *moxF* upstream region from *Methylobacterium extorquens* AM1rif. They constructed a transcriptional fusion between the *lacZ* gene and a 1.55 kb fragment containing the 5' portion of the *moxF* gene and its 1289 bp upstream DNA. In our study, we cloned a 448 bp DNA fragment upstream of *moxF* (not including any *moxF* structural sequence) in front of *xylE* gene and demonstrated that this 448 bp fragment contained necessary elements for methanol-inducible regulation when carried by a plasmid. Interestingly, the transcriptional fusion described by Morris & Lidstrom (1992) was not regulated by methanol when carried on the vector. The fusion was normally regulated by methanol once it was integrated into the chromosome (Morris & Lidstrom, 1992).

In the present study, we have described the construction of a promoter-probe vector which uses *xylE* as the reporter gene. The vector was successfully employed to detect methanol-regulated DNA fragments from *M. organophilum* XX that promote the expression of *xylE* *in vivo*. In particular, this vector enabled us to identify a divergently transcribed gene (*moxW*) upstream of *moxF*. The *moxW* gene was also shown to be regulated by methanol. This is significant because it demonstrated that vector pHX200 is a very efficient promoter-probe vector. Besides identification of methanol-regulated promoters, vector pHX200 can also be used to identify

other types of promoter fragments such as succinate-regulated, or general 'housekeeping' promoters from methylotrophic bacteria by observing the expression of promoter clones under different growth conditions.

In both *M. organophilum* XX and *M. extorquens* AM1, methanol oxidation is not repressed by the presence of multi-carbon compounds (Anthony, 1982; O'Connor, 1981). Fifteen genes that are involved in methanol oxidation in *M. organophilum* XX have been identified (Allen & Hanson, 1985; Machlin *et al.*, 1987, 1988). Seventeen genes were found to be involved in methanol oxidation in *M. extorquens* AM1 (Nunn & Lidstrom, 1986a, b; Lidstrom, 1990). Most genes have homologues in these two species and in *Methylosinus* sp. 81Z (Bastien *et al.*, 1989). The functions of several of these genes are not known. Genes involved in methanol oxidation can be divided into two categories. One includes genes that are involved in regulation of *moxF* transcription (they are called 'pre *moxF*-regulation genes'). The other category contains genes that are either structural genes for MDH and MDH-specific electron-carrier proteins or possess post-translational functions such as addition of the prosthetic group PQQ to the MDH apoprotein, transport of MDH to the periplasm, etc. (these are termed 'post *moxF*-regulation genes'). Mutations in these two types of genes can be differentiated by introducing plasmid pHX200V-47 (containing *moxFp::xylE* fusion) into each *M. organophilum* XX mutant strain and measuring *xylE* expression after growing these mutants in a succinate minimal medium supplemented with methanol as an inducer. It should be pointed out that succinate does not decrease the specific activity of MDH when methanol is present in the growth medium (O'Connor, 1981). Mutants with mutations in genes of the post *moxF*-regulation category are able to synthesize active *xylE* protein and produce a yellow colour after addition of catechol, because the *xylE* product does not require PQQ, cytochrome *c_L* or the small MDH subunit for activity, nor does it require a transport system for activity. In contrast, regulatory mutants of pre *moxF*-regulation genes where products are required for transcription of *moxF* cannot produce catechol 2,3-dioxygenase, because the *xylE* gene on the plasmid is controlled by the same mechanism as the chromosomal *moxF* gene and each regulatory mutation is defective in one of the steps prior to the activation of the *moxF* promoter. This assay therefore categorized *moxB*, *moxQ*, *moxE*, *moxM*, *moxN* and *moxD* as pre *moxF*-regulation loci and *moxA*, *moxK*, *moxL*, *moxI*, *moxF*, *moxO* and *moxC* as post *moxF*-regulation genes.

It is logical to see that mutations which led to defective MDH large and small subunits did not interfere with the promoter activity of the *moxF* gene. The results of this study also confirmed the roles for the *moxQ* and *moxE*

genes suggested previously (Machlin *et al.*, 1987; Nunn & Lidstrom, 1986b) and supported the proposed roles of *moxA*, *moxK* and *moxL* (Lidstrom, 1990). Morris & Lidstrom (1992) also introduced their transcriptional fusion into *moxB* mutants (UV4rif, UV25rif) to observe the effects of the mutation on the gene expression controlled by the 1.55 kb promoter fragment. In these mutants, *lacZ* expression was reduced to the background level when the fusion was present in both plasmid and chromosomal locations. Their observation was consistent with our results that activity of *moxF* promoter was totally shut down by the mutations in locus *moxB* of mutants UV4, UV25 and SM37.

Inclusion of other methanol-regulated transcriptional fusions in this study enabled us to differentiate two types of regulatory genes. Mutations in the *moxM*, *moxN* and *moxD* genes blocked expression of all three transcriptional fusions (*moxFp::xylE*, *moxWp::xylE* and *moxUp::xylE*). This suggests that these three methanol-regulated genes share the same induction mechanism. In contrast, mutations of the *moxB*, *moxQ* and *moxE* genes only inhibited the *moxFp::xylE* fusion but not the other two, indicating that the *moxF* gene has additional activation mechanisms that are governed by the *moxB*, *moxQ* and *moxE* genes. These results showed that the transcriptional fusion system using *xylE* as reporter gene is a very efficient tool for categorizing methanol oxidation genes of *M. organophilum* XX and *M. extorquens* AM1.

In *M. organophilum* XX, both the *moxF* and *moxW* promoters deviate significantly from the consensus sequence and have very poor recognition sites for RNA polymerase, suggesting positive regulation for both genes. These two promoters are both inducible by methanol. Their transcription start sites have been identified and are 143 bp apart. In between these two start sites, there are three identical copies of a septanucleotide (AGAAATG), which has also been found in other newly identified methanol-regulated promoter sequences in relatively conserved form (H. H. Xu and others, unpublished data). Several copies of this septanucleotide are also present upstream of the *moxF* gene from *M. extorquens* strain AM1 (Anderson *et al.*, 1990). Deletional analysis indicated that removal of a nucleotide segment containing the distal (relative to *moxF* transcription start) copy of the septanucleotide (see Fig. 4) totally abolished *moxFp* activity (H. H. Xu and others, unpublished data). There is a dyad symmetry (TGTCT-N₁₀-AGACA) overlapping with the -35 region of the *moxF* promoter (see Fig. 4). This symmetry resembles a consensus sequence (TGTGT-N₆₋₁₀-ACACA) of prokaryotic positive regulator binding sites (Gicquel-Sanzey & Cossart, 1982). DNAase I footprinting experiments have indicated that this symmetry was protected against

DNAase I nicking after binding to the putative regulatory protein(s) (H. H. Xu and others, unpublished data).

We propose that *moxM*, *moxN* and *moxD* are general positive regulatory genes for a group of methanol-inducible genes (such as *moxF*, *moxW* and *moxU*). One of these regulatory genes may encode a DNA-binding protein capable of binding to the conserved septanucleotides in the upstream region of target genes. The *moxF* gene is also positively controlled by *moxB*, *moxQ* and *moxE* genes, which have no influence on the expression of *moxW* and *moxU*. One of these regulatory genes may encode a DNA-binding protein whose binding site is the dyad symmetry TGTCT-N₁₀-AGACA.

This work was supported by a grant from the US Department of Energy (DOE-F602-88ER 13682). The *E. coli* strain containing pTG402 was kindly provided by Dr J. P. Lecocq of Transgene, Strasbourg, France. The mutants of *Methylobacterium extorquens* AM1 were gifts from D. Nunn and M. E. Lidstrom of California Institute of Technology, Pasadena, California, USA. We thank Dr Terese Barta for technical help and advice.

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