The *mxoAKL* genes of *Methylobacter albus* BG8

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The facultative methanol utilizer *Methylobacterium extorquens* AM1 contains at least three genes (*mxoA*, *K* and *L*) that encode functions involved in providing calcium to the holoenzyme of methanol dehydrogenase, the enzyme that oxidizes methanol to formaldehyde in this strain. Methane-utilizing bacteria (methanotrophs) also contain methanol dehydrogenase, and evidence suggests that similar methanol oxidation (Mox) functions may be present in some of these strains. DNA fragments from *Methylobacterium extorquens* AM1 specific to *mxoA*, *mxoK* and *mxoL* were isolated for use as hybridization probes against genomic digests of a variety of methanotrophic bacteria. Only the *mxoL* probe showed substantial hybridization, and it was used to identify and isolate an 8.5 kb *HindIII* fragment from *Methylobacter albus* BG8 (a Type I methanotroph). Hybridization of restriction digests of this fragment to individual probes for *Methylobacterium extorquens* AM1 *mxoA*, *K* and *L* indicated that the relative *mxo* gene order in *Methylobacter albus* BG8 is *A–K–L*. A T7 dual promoter/polymerase protein expression system indicated that five polypeptides are expressed from a 4.5 kb region of *Methylobacter albus* BG8 DNA in *Escherichia coli*, all transcribed in the same direction, and they apparently correspond to *mxoACKDL*. The functions of *mxoC* and *mxoD* are currently not known, but the order of *mxoDL* is reversed in *Methylobacter albus* BG8 compared to *Methylobacterium extorquens* AM1. When subclones of the *Methylobacter albus* BG8 fragment containing these genes were used as hybridization probes to genomic digests of methanotrophic bacteria, specific bands were detected that suggested a similar gene order in most cases. These data indicate that the *mxoAKL* region is relatively highly conserved in methanotrophs, and that in most cases the *mxoAKL* genes are grouped together in the same order as in the facultative methanol utilizer *Methylobacterium extorquens* AM1.

Keywords: methanotroph, *mox* genes, calcium insertion, methanol oxidation, *Methylobacter albus*

INTRODUCTION

In Gram-negative methylotrophic bacteria, methanol is oxidized to formaldehyde by the periplasmic quinoprotein

methanol dehydrogenase (Anthony, 1986). Methanol dehydrogenase (MDH) consists of two large and two small subunits and two molecules of the cofactor pyrroloquinoline quinone (PQQ), which are noncovalently attached to each of the large subunits. In addition, each PQQ site contains a calcium ion, which is apparently involved in maintaining PQQ in the correct configuration (Richardson & Anthony, 1992; Anthony *et al.*, 1994; White *et al.*, 1993). This enzyme has been shown to be conserved biochemically and antigenically in a variety of methylotrophic bacteria (Anthony, 1986) and at the nucleotide sequence level in three different methanol utilizers (Anderson *et al.*, 1990; Harms *et al.*, 1987; Machlin & Hanson, 1988). Recent evidence shows that at least 24 complementation groups are involved in the

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

The GenBank accession number for the *Methylobacterium extorquens* AM1 *mxoACKDL* sequence is L41608.

oxidation of methanol to formaldehyde (Mox functions) in the facultative methanol utilizer *Methylobacterium extorquens* AM1 (Anderson & Lidstrom, 1988; Lidstrom, 1988; Nunn & Lidstrom, 1986a, b; Lidstrom *et al.*, 1994) and at least 13 in *Methylobacterium organophilum* XX (Bastien *et al.*, 1989; Machlin *et al.*, 1988).

Three of the known Mox complementation groups define *mxmA*, *K* and *L*, which are located immediately 3' to a cluster of *mxg* genes (*mxgFJGIRS*) in *Methylobacterium extorquens* AM1 (Nunn & Lidstrom, 1986a; Lidstrom *et al.*, 1994; Morris *et al.*, 1995). The *mxmA*, *K* and *L* genes are adjacent on the chromosome and their gene products are involved in the acquisition of calcium ions by the holoenzyme (Richardson & Anthony, 1992). MDH from mutants of all three classes is inactive, no longer contains calcium, and has an altered absorption spectrum. When the inactive MDH from these mutants was incubated in calcium salts, the full enzyme activity returned over time and the absorption spectrum corresponding to that of fully reduced wild-type MDH was concomitantly restored (Richardson & Anthony, 1992). Therefore, the *mxmA*, *K* and *L* gene products may be calcium-binding proteins, may be involved in maintaining a high calcium concentration in the periplasm, or may stabilize a configuration of MDH that permits incorporation of low calcium concentrations into the protein. Two other genes (*mxgC* and *mxgD*) are known to be present in this region, but their function is not yet known (Morris *et al.*, 1995). At this time, it appears that *mxgAKL* in *Methylobacterium extorquens* AM1 are part of a large gene cluster that includes (in order) *mxgFJGIRSAKCLD*.

The Mox system is clearly complex in facultative methanol utilizers and it is important to determine whether a similar complexity exists in other methylotrophs at both functional and genetic levels. We are particularly interested in the obligate methane-utilizing bacteria (methanotrophs) due to their pivotal role in both commercial (Hou, 1984) and environmental (Thompson & Cicerone, 1986) processes. Data exist suggesting that the Mox system in methanotrophs shows functional similarity to the Mox system in facultative methanol utilizers, since Mox genes from methanotrophs can complement Mox mutations in *Methylobacterium* strains (Stephens *et al.*, 1988; Bastien *et al.*, 1989).

The purpose of the present study was to isolate and characterize *mxmA*, *K* and *L* genes from a Type I methanotroph. A gene probe derived from cloned *Methylobacterium extorquens* AM1 DNA containing *mxgL* was used to identify specific hybridizing fragments in genomic digests of DNA from a variety of obligate methanotrophs, and to clone *mxmA*, *K* and *L* from *Methylobacter albus* BG8. Expression studies suggested that in *Methylobacter albus* BG8 this hybridizing region contains at least five genes, equivalent to *mxgACKLD* of *Methylobacterium extorquens* AM1. Functional complementation of *Methylobacterium extorquens* AM1 *MxA*, *K* and *L* mutants was not achieved with any of the *Methylobacter albus* BG8 fragments tested.

METHODS

Bacterial strains, plasmids and phage. The bacterial strains, plasmids and bacteriophage used in this study are listed in Table 1.

Media and growth conditions. *Methylobacterium extorquens* AM1 was grown at 30 °C in the ammonium/mineral salts medium described by Harder *et al.* (1973) supplemented with a vitamin mixture (Staley, 1981) or in nutrient broth (Difco). *Escherichia coli* strains were grown at 37 °C in Luria broth (Maniatis *et al.*, 1982). Filter-sterilized supplements were added to media as follows: 0.2% (w/v) succinate; 0.5% (v/v) methanol; 12.5 µg tetracycline ml⁻¹; 10 µg rifampicin ml⁻¹; 50 µg kanamycin ml⁻¹; and 50 µg ampicillin ml⁻¹. Methanotrophs were grown at 30 °C, except *Methylobacter* sp. strains A1, A45 and MN, which were grown at 37 °C and *Methylococcus capsulatus* strain Bath, which was grown at 42 °C. All methanotrophs were grown in the nitrate mineral salts medium described by Whittenbury & Dalton (1981) supplemented with a vitamin mixture (Staley, 1981). Methanotrophs were maintained on plates containing medium plus 1.5% (w/v) Bacto-Agar (Difco) in gas canisters under a methane:air atmosphere (1:1) and were grown in liquid culture with shaking under an atmosphere of the same composition.

Matings for complementation. Plasmids (pRK310 containing appropriate *Methylobacter albus* BG8 DNA inserts or *Methylobacterium extorquens* DNA inserts as controls) were mobilized into *Methylobacterium extorquens* AM1 in three-way matings as described previously (Stephens *et al.*, 1988), except that *Methylobacterium extorquens* strains were taken from fresh agar plates. Additionally, the admixture of strains was spotted directly onto nutrient agar plates instead of filters for overnight incubation.

DNA manipulations. Plasmid DNA was isolated from *E. coli* either by the rapid screening method of Holmes & Quigley (1981) or the large-scale method of Ish-Horowitz & Burke (1981). Plasmid DNA from large-scale preparations was further purified by two rounds of banding in CsCl/ethidium bromide density gradients.

Chromosomal DNA was prepared by the method of Marmur (1961), scaled down to a 1.5 ml volume.

Restriction enzymes were obtained from BRL or New England Biolabs and were used according to the manufacturers' specifications. Most analytical restriction digests were performed overnight in the presence of RNaseA to ensure complete digestion of the DNA. Chromosomal DNA digests were typically carried out for 4 h prior to separation and hybridization.

Agarose gel electrophoresis purification of DNA fragments and DNA ligations and transformations of *E. coli* strains were performed as described by Maniatis *et al.* (1982).

Radiolabelling of DNA was performed by the nick-translation procedure of Rigby *et al.* (1977) or by a random-priming procedure using the specifications of the supplier (Boehringer Mannheim).

DNA-DNA hybridization. Hybridizations were performed using dried agarose gels as described by Meinkoth & Wahl (1984). Hybridization incubations for blots containing genomic DNA were carried out at 53 °C in 6 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% (w/v) SDS, 0.5% (w/v) non-fat dried milk and 0.01 M EDTA (pH 8.0) followed by four 15 min washes in 2 × SSC and 0.1% SDS, two at room temperature and two at 53 °C. For probing of cloned fragments, each lane on the agarose gel was loaded with approximately 1.0 µg *Methylobacter albus* BG8 DNA (5.9 kb *EcoRI* fragment or 8.5 kb *HindIII* fragment) digested with the designated re-

Table 1. Bacterial strains and plasmids used in this study

Strains	Relevant traits	Source/reference
<i>E. coli</i>		
DH5 α	$r^- m^+ recA1 \phi 80dlacZ\Delta M15 (lacZYA-argF)U169 hsdR$	BRL
HB101	$r^- m^- hsdS recA13 ara-14 supE lacY mcrB galK proA xyl-5 mtl-1 leu-1 rpsL$	Boyer & Roulland-Dussoix (1969)
Methanol utilizers		
<i>Methylobacterium extorquens</i> AM1		
AM1 rif	Rif ^r -derivative	Nunn & Lidstrom (1986a)
AM1 PG1	MxaA mutant of AM1, rif ^r	Nunn & Lidstrom (1986a)
AM1 UV4	MxaK mutant of AM1, rif ^r	Nunn & Lidstrom (1986a)
AM1 M15a	MxaL mutant of AM1, rif ^r	Nunn & Lidstrom (1986a)
Methane utilizers		
Type I		
<i>Methylobacter albus</i> BG8		Whittenbury <i>et al.</i> (1970); Bowman <i>et al.</i> (1993)
<i>Methylobacter</i> sp. strain A1		Lidstrom (1988)
<i>Methylobacter marinus</i> strain A4		Lidstrom (1988); Bowman <i>et al.</i> (1993)
<i>Methylomonas</i> sp. strain MN		This laboratory
<i>Methylobacter capsulatus</i> Y		Whittenbury <i>et al.</i> (1970)
Type II		
<i>Methylosinus trichosporium</i> OB3b		Whittenbury <i>et al.</i> (1970)
<i>Methylosinus sporium</i> 5		Whittenbury <i>et al.</i> (1970)
<i>Methylocystis parvus</i> OBBP		Whittenbury <i>et al.</i> (1970)
<i>Methylocystis</i> sp. strain LWY		This laboratory
Type X		
<i>Methylococcus capsulatus</i> Bath		Whittenbury <i>et al.</i> (1970)
Plasmids		
pVK100	Tc ^r Km ^r IncPI cosmid	Knauf & Nester (1982)
pRK310	Tc ^r <i>lacPOZ'</i> IncPI	Ditta <i>et al.</i> (1985)
pRK2013	Km ^r -mobilizing plasmid	Figurski & Helinski (1979)
Plasmids containing inserts of <i>Methylobacterium extorquens</i> AM1 DNA		
pDN9	pVK100 (4.4 kb <i>Hind</i> III- <i>Xba</i> I); <i>mxaA</i> , K, L	Nunn & Lidstrom (1986a)
pYK310	pRK310 (0.9 kb <i>Pst</i> I- <i>Bam</i> HI); <i>mxaA</i>	
pCT2	pRK310 (0.9 kb <i>Sac</i> II- <i>Eco</i> RI); <i>mxaK</i>	
pCT3	pRK310 (1.8 kb <i>Sal</i> I); <i>mxaL</i>	
Plasmids containing inserts of <i>Methylobacter albus</i> BG8 DNA		
pYK-B25	pVK100 (25 kb <i>Hind</i> III)	This study
pYK-BH1	pRK310 (8.5 kb <i>Hind</i> III)	
pYK-BE2	pRK310 (5.9 kb <i>Eco</i> RI)	
pBMX834	pT76 (1.4 kb <i>Bam</i> HI- <i>Nru</i> I)	
pBMX854	pT76 (3.2 kb <i>Bam</i> HI- <i>Pst</i> I)	
pBMX856	pT76 (3.5 kb <i>Bam</i> HI- <i>Eco</i> RI)	
pBMX860	pT76 (4.0 kb <i>Bam</i> HI- <i>Bam</i> HI)	
pBMX854 Δ BN	pBMX854 with deletion of 1.4 kb <i>Bam</i> HI- <i>Nru</i> I	
pBMX860 Δ BN	pBMX860 with deletion of 1.4 kb <i>Bam</i> HI- <i>Nru</i> I	
pYK-HH1	pRK310 (8.5 kb <i>Hind</i> III), orientation right to left (<i>plac</i>)	
pYK-HH4	pRK310 (8.5 kb <i>Hind</i> III), orientation left to right (<i>plac</i>)	
pYK-EE1	pRK310 (5.9 kb <i>Eco</i> RI), orientation right to left (<i>plac</i>)	
pYK-EE5	pRK310 (5.9 kb <i>Eco</i> RI), orientation left to right (<i>plac</i>)	
pCT155	pRK310 (4.0 kb <i>Bam</i> HI), orientation right to left (<i>plac</i>)	
pCT153	pRK310 (4.0 kb <i>Bam</i> HI), orientation left to right (<i>plac</i>)	
pCT158	pRK310 (3.5 kb <i>Pst</i> I- <i>Hind</i> III), orientation right to left (<i>plac</i>)	
pCT157	pRK310 (0.3 kb <i>Pst</i> I- <i>Eco</i> RI), orientation left to right (<i>plac</i>)	

striction enzyme. Control lanes (either *Methylobacterium extorquens* probe DNA alone or pUC19 plasmid DNA containing probe DNA, digested to yield the probe insert) were loaded to give approximately 10 ng probe DNA. Hybridization incubations in these cases were as described above except they were

carried out at 42 °C. Washes were performed as follows: once at room temperature followed by once at 53 °C or at 59 °C. Colony hybridizations were carried out by the procedure described by Maniatis *et al.* (1982) using 1–5 $\times 10^6$ c.p.m. of ³²P-labelled probe DNA.

Protein expression. Protein expression from cloned genes was accomplished using the T7 promoter vectors pT7-5 and pT7-6 and plasmid pGP1-2 containing the T7 polymerase gene, as described by Tabor (1987). Promoter vectors pT7-5 and pT7-6 each contain a polylinker cloning region downstream of the T7 promoter followed by the ampicillin-resistance gene, which is transcribed in the opposite orientation to the T7 promoter. Each *Methylobacter albus* BG8 DNA insert was cloned and tested in both orientations with respect to the T7 promoter.

RESULTS

Genomic probing with the *mxmA*, *K* and *L* genes

In order to determine whether similarity exists among the *mxmA*, *K* and *L* genes of *Methylobacterium extorquens* AM1 and chromosomal DNA obtained from obligate methanotrophs, DNA fragments containing each of these genes were isolated from *Methylobacterium extorquens* AM1 to use as hybridization probes. The fragments selected were a 0.9 kb *Pst*I–*Bam*HI fragment containing *mxmA*, a 0.9 kb *Sac*II–*Eco*RI fragment containing *mxmK*, and a 1.8 kb *Sa*I–*Sa*I fragment containing *mxmL* (Fig. 1a; Nunn & Lidstrom, 1986a). The latter fragment is now known to contain *mxmD* also (Morris *et al.*, 1995).

Each of these isolated fragments was used to probe *Eco*RI digests of genomic DNA from several different methanotrophs representing all three major groups (Type I, Type II and Type X). In each case, a variety of stringency

Fig. 2. Hybridization of the isolated *Methylobacterium extorquens* AM1 *mxmLD* gene probe to DNA from methanotrophs. Total genomic DNA was digested with *Eco*RI, separated on agarose gels and dried. Each lane contained approximately 1.5 µg total DNA. The dried gels were hybridized to ³²P-labelled probe DNA (isolated fragment) and exposed to X-ray film. Conditions of hybridization allowed approximately 35% base pair mismatch. Lanes: 1, *Methylocystis* sp. strain; 2, *Methylosinus trichosporium* OB3b; 3, *Methylocystis parvus* OBBP; 4, *Methylomonas* sp. strain MN; 5, *Methylobacter capsulatus* Y; 6, *Methylobacter marinus* A45; 7, *Methylococcus capsulatus* Bath; 8, *Methylobacter albus* BG8; 9, *Methylosinus sporium* 5. Sizes of the major hybridizing bands are indicated.

conditions were tested, but conditions allowing 30–37% base pair mismatch (see Methods) produced the clearest results. With the *mxmA* and *mxmK* probes, only weak hybridization to specific bands was detected under all conditions tested (data not shown). However, much stronger hybridization was observed in most cases using the *mxmLD* probe (Fig. 2). The approximate sizes of the major hybridizing bands are indicated in Fig. 2. Hybridization was detected for two Type I strains, all of the Type II strains and the Type X strain, but no significant hybridization was detected for two of the Type I strains (*Methylomonas* sp. MN and *Methylobacter capsulatus* Y) (see Fig. 2). Positive controls containing *Methylobacterium extorquens* AM1 genomic DNA identified the predicted *Eco*RI fragments for each probe and negative controls (*E. coli* and bacteriophage lambda) showed no hybridization (data not shown).

Cloning of the *mxm* genes from *Methylobacter albus* BG8

The data presented above suggest that significant similarity of the *mxmLD* genes occurs in diverse methanotrophs and some conservation of sequences may also be present in *mxmA* and *K* genes. In order to determine whether the hybridization observed was due to the presence of true *mxmA*, *K* and *L* homologues, we cloned the corresponding DNA fragments from one of the methanotrophs. *Methylobacter albus* BG8 was chosen, since the *mxmLD* probe showed good hybridization to *Methylobacter albus* BG8 genomic DNA (Fig. 2), and a genomic clone bank (a *Hind*III partial digest of *Methylobacter albus* BG8 DNA ligated to the broad-host-range cosmid vector pVK100) is available for this strain (Stephens *et al.*, 1988). Colony blots of this bank were screened for hybridization to the *mxmLD* probe, and positive clones were identified.

(a) *Methylobacterium extorquens* AM1

(b) *Methylobacter albus* BG8

Fig. 1. *mxmA*KL regions of two methanotrophs. The patterned boxes denote the restriction fragments used for hybridization probes. (a) *Methylobacterium extorquens* AM1 region showing the location of *mxmA*, *C*, *K*, *L* and *D*. The arrow above each gene shows the direction of transcription. (b) The *Methylobacter albus* BG8 insert in pYK-BH1. The lines underneath show the minimum regions that hybridized to the corresponding probes in (a), with the thin lines representing light hybridization. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; N, *Nru*I; P, *Pst*I; S, *Sa*I; Sc, *Sac*II; X, *Xho*I.

Fig. 3. Hybridization of the cloned *Methylobacter albus* BG8 4.0 kb *Bam*HI fragment containing the *mxoAKL* region to *Methylobacter albus* BG8 chromosomal DNA digested with different enzymes. Lanes: 1, *Bam*HI; 2, *Hind*III; 3, *Eco*RI. Sizes of the hybridizing bands are indicated.

Each of these clones contained an identical set of *Hind*III fragments of approximately 8.5, 7.0, 3.4, 3.2, 1.4 and 1.3 kb in size. The *mxoLD* probe hybridized specifically to the 8.5 kb fragment. This fragment was subcloned into the broad-host-range vector pRK310 and the insert was mapped using a variety of restriction enzymes (Fig. 1b). This 8.5 kb region included a 5.9 kb *Eco*RI (E_1 – E_2) fragment as identified in the initial genomic blots (Fig. 2). When blots of genomic *Eco*RI, *Hind*III and *Bam*HI digests were tested with the 4 kb *Bam*HI *Methylobacter albus* BG8

fragment (B_1 – B_2 in Fig. 1b) as a probe, restriction fragments of approximately 5.9, 8.5 and 4.0 kb were detected, respectively (Fig. 3), confirming that the cloned fragment was present in the chromosome of *Methylobacter albus* BG8.

Identification of the *mxoAKL* regions

Each of the *Methylobacterium extorquens* AM1 *mxo* probes was used for hybridization to restriction digests of the 8.5 kb *Hind*III fragment or an appropriate subclone in order to delineate the position of each of the *mxo* genes in *Methylobacter albus* BG8. The *mxoLD* probe hybridized most strongly to a 0.8 kb *Pst*I–*Bam*HI (P_3 – B_2) fragment and more weakly to the adjacent 3.3 kb *Bam*HI–*Pst*I (B_1 – P_3) fragment (Figs 1 and 4a). The cloned 5.9 kb *Eco*RI and 8.5 kb *Hind*III fragments were probed with the *Methylobacterium extorquens* AM1 *mxoA* and *mxoK* probes, respectively, and in both cases, the strongest region of hybridization was to the 3.3 kb *Bam*HI–*Pst*I (B_1 – P_3) and the 4.0 kb *Pst*I–*Pst*I (P_2 – P_3) fragments located to the left of the *mxoLD*-hybridizing region (Figs 1 and 4b, c). The *mxoA* probe also hybridized weakly to the adjacent 1.2 kb *Sal*I–*Bam*HI region (S_1 – B_1), and the *mxoK* probe hybridized more strongly to the 3.4 kb *Nru*I–*Nru*I (N_1 – N_2) fragment than the adjacent 4 kb *Hind*III–*Nru*I (H_1 – N_1) fragment. These data suggested that the region similar to *mxoLD* is located to the right of the region similar to *mxoA* and *mxoK*, and the region similar to *mxoK* is

Fig. 4. Hybridization of the *Methylobacterium extorquens* AM1 *mxo* gene probes to restriction digests of the 8.5 kb *Hind*III or the 5.9 kb *Eco*RI *Methylobacter albus* BG8 fragments that hybridize to the *mxoLD* probe. Lanes denoted 'probe' contained purified probe DNA in each case. Conditions of hybridization allowed approximately 30% base pair mismatch (a) or 37% bp mismatch (b and c). (a) *mxoLD* probe, digestions of the 8.5 kb *Hind*III fragment; (b) *mxoA* probe, digestions of the 5.9 kb *Eco*RI fragment; (c) *mxoK* probe, digestions of the 8.5 kb *Hind*III fragment. In each case, the larger *Pst*I fragment identified is P_2 – P_3 (4.0 kb) and the larger *Sal*I fragment identified is S_2 – S_3 (3.35 kb).

Fig. 5. Autoradiogram of polypeptides from whole-cell extracts labelled with [35 S]methionine using the T7 polymerase/promoter expression system and electrophoresed using 12% SDS-PAGE. *E. coli* DH5 α was the host for all constructs, and cells were induced for 20 min. All fragment designations refer to Fig. 6. (a) The first two lanes are vector controls with pT7-5 and pT7-6, respectively. The other lanes represent constructs of pT7-5 or pT7-6 containing the designated fragments, oriented left to right as shown in Fig. 6 with respect to the T7 promoter. Lanes: 3, pBMX834; 4, pBMX854; 5, pBMX856; 6, pBMX860; 7, pBMX854 Δ BN; 8, pBMX860 Δ BN. (b) Lanes: 1, pT7-6; 2, pCT120; 3, pCT110. Molecular mass standards (kDa) are indicated by bars and the insert-specific polypeptides are indicated by arrows.

located to the right of that similar to *mxoA*, as shown in Fig. 1b. No significant hybridization was observed with the *mxoA* or *mxoK* probe to the 0.8 kb *Pst*I–*Bam*HI (P_3 – B_2) fragment that hybridized to the *mxoLD* probe. As shown in Fig. 4(c), hybridization of the *mxoK* probe to these fragments consistently yielded fuzzy bands. In order to identify the hybridizing fragments it was necessary to compare several exposures to the original ethidium bromide stained gels. One of these is shown in Fig. 4(c).

Expression studies. In order to further analyse the *Methylobacter albus* BG8 region containing the putative *mxoA*, *K* and *L* genes, the dual T7 promoter/polymerase system described by Tabor (1987) was used to determine the direction of transcription and the size and location of the gene products. A variety of subclones of the 4.0 kb *Bam*HI (B_1 – B_2) fragment containing the region that hybridized to the *mxoA*, *K* and *LD* probes were tested for expression in *E. coli*, and a total of seven polypeptides were consistently observed (Figs 5a and 6), all transcribed left to right as shown in Fig. 6. All seven of the polypeptides (38, 33, 32, 29.5, 28, 26.5, and 20 kDa) were expressed from the *Bam*HI (B_1 – B_2) fragment. Two sets of

these (33/32 and 29.5/28 kDa) were always observed as pairs, and are probably either unprocessed and mature forms of secreted polypeptides or the result of multiple start sites for the same polypeptide, as has been observed previously in this expression system (Chistoserdov *et al.*, 1991). In some cases, as for pBMX834, insufficient coding region is available to encode two polypeptides of 33/32 kDa, further supporting this conclusion. It has been proposed that at least some of the *mxoAKL* functions should be periplasmic (Nunn & Lidstrom, 1986b; Richardson & Anthony, 1992), which supports the idea that these double bands may represent unprocessed and mature forms of secreted polypeptides.

The 33/32 kDa polypeptides were expressed from all clones containing the 1.4 kb *Bam*HI–*Nru*I (B_1 – N_1) fragment, which is the region that hybridized most strongly to the *mxoA* probe. The 38 kDa polypeptide was only expressed from the plasmids containing DNA across the N_1 *Nru*I site, suggesting that the coding region for this polypeptide lies across this site. This coding region must initiate within a few tenths of a kb upstream of this site, to allow room for encoding the 33/32 kDa polypeptide

Fig. 6. Map of the clones used in the T7 expression experiments and the polypeptides observed from each. In all cases, the polypeptides were only observed when the indicated fragments were cloned into the appropriate expression vector such that the orientation with respect to the T7 promoter was left to right, as shown. Restriction sites are the same as in Fig. 1(b). The 29.5/28 kDa pair of polypeptides appear to be truncated forms of the 38.5 kDa polypeptide (see text).

upstream. Preliminary sequencing of the region around the N_1 *NruI* site revealed a partial ORF with 39% identity to the *Methylobacterium extorquens* AM1 *mxsA* across a 118 amino acid overlap (A. Springer & M. Lidstrom, unpublished data), suggesting that the 38 kDa polypeptide corresponded to MxA. The 26.5 and 20 kDa polypeptides were expressed from all clones containing the 1.6 kb *NruI*-*PstI* (N_1 - P_3) fragment, just downstream of the region encoding the 33/32 kDa polypeptides. This was the region that hybridized most strongly to the *mxsK* probe, but it also hybridized to the *mxsL* probe. The region from the B_1 site to the P_3 site contains just enough coding potential for all of these polypeptides, assuming that the 33/32 kDa polypeptides are encoded by the same gene. The 29.5/28 kDa polypeptides were expressed from both clones containing the complete *NruI*-*BamHI* (N_1 - B_2) fragment that includes DNA downstream of the region encoding the 26.5 and 20 kDa polypeptides. This downstream region hybridized most strongly to the *mxsL* probe. In addition, three other poorly expressed polypeptides of 21, 17.5 and 15.5 kDa showed the same pattern of expression as the 29.5/28 kDa polypeptides. However, none of these were expressed from clones missing the right-hand 0.5 kb *EcoRI*-*BamHI* (E_2 - B_2) or 0.8 kb *PstI*-*BamHI* (P_3 - B_2) fragments. This indicates that none of these polypeptides are wholly encoded upstream of the *EcoRI* site denoted E_2 on Fig. 6. These data strongly suggest that the smaller three polypeptides are artefacts, either breakdown products or the result of internal start sites, and that the 29.5/28 kDa pair are encoded by a gene that has a start located in the region of the *PstI* site denoted P_3 in Fig. 6. Preliminary sequencing upstream of the E_2 site revealed a partial ORF that showed 31% identity to *mxsL* of *Methylobacterium extorquens* AM1 across 39 amino acids (A. Springer & M. Lidstrom, unpublished data). In *Methylobacterium extorquens* AM1, *mxsL* is predicted to encode a 37 kDa polypeptide, suggesting that this gene might extend across

the B_2 site. In order to test this hypothesis, two other subclones were used for expression that include DNA past that site to the *HindIII* site (H_2) (Figs 5b and 6). In both cases, none of the polypeptides observed from the B_1 - B_2 clones were present. For the P_3 - H_2 subclone, two new polypeptides of 38.5 and 23.5 kDa were present, while in the smaller B_2 - H_2 subclone, only the 23.5 kDa polypeptide was present (Fig. 5b). These data suggest that the 29.5/28 kDa polypeptides represent truncated products of a gene equivalent to *mxsL*, that crosses the B_2 site and encodes a 38.5 kDa polypeptide. The 23.5 kDa polypeptide must be encoded downstream of the 38.5 kDa polypeptide.

Complementation of *Methylobacterium extorquens* AM1 MxA, K and L mutants

The results presented above suggest that *Methylobacter albus* BG8 contains genes equivalent to the *mxsA*, K and L genes of *Methylobacterium extorquens* AM1. Having cloned and localized these *mxs* genes and also shown their expression in *E. coli*, we attempted to complement the MxA, K and L mutants of *Methylobacterium extorquens* AM1 using various constructs of *Methylobacter albus* DNA in the broad-host-range vector pRK310. These included the larger *HindIII*, *EcoRI* and *BamHI* fragments (H_1 - H_2 , E_1 - E_2 , B_1 - B_2), cloned in both orientations, the *PstI*-*HindIII* (P_3 - H_2) fragment cloned right to left with respect to the *lac* promoter in pRK310 and the *PstI*-*EcoRI* (P_3 - E_2) fragments cloned left to right with respect to the *lac* promoter in pRK310 (Fig. 1). These clones were mated into the appropriate *Methylobacterium extorquens* AM1 MxA, K and L mutants and after a 2-week incubation, colonies containing the test clones were compared to controls. Positive controls were wild-type and mutants complemented with the appropriate *Methylobacterium extorquens* AM1 clones, and negative controls were the mutants containing vector alone. No growth was observed for

Fig. 7. Hybridization of the isolated *Methylobacter albus* BG8 *mxmA*, *CK* and *L* probes to DNA from methanotrophs. Probes: (a) *mxmA*; (b) *mxmCK*; (c) *mxmL*. Lanes: 1, *Methylobacter albus* BG8; 2, *Methylococcus capsulatus* Bath; 3, *Methylocystis parvus* OBBP; 4, *Methylobacter marinus* A45. Sizes of standards are shown on the right. Chromosomal DNA was digested with *Bst*YI.

cells containing the test clones above that seen with the negative controls. In all cases, the same mating mixtures plated onto medium containing succinate plus tetracycline produced a large number of colonies as compared to negative controls with no vector, confirming that plasmid transfer had occurred.

Hybridization to methanotroph DNA

Three subclones of the *Methylobacter albus* BG8 *Bam*HI fragment containing the putative *mxmA*, *CK* and *L* regions were used as hybridization probes to genomic digests of DNA from three of the methanotrophs that showed hybridization to the *Methylobacterium extorquens* AM1 *mxmL* probe. The three subclones were *B*₁-*N*₁, which should contain mainly *mxmA*, *N*₁-*P*₃, which should contain *mxmK* and the major portion of *mxmC*, and *P*₃-*B*₂, which should be specific to *mxmL* (Fig. 1). The strains tested were *Methylococcus capsulatus* Bath, a Type X strain, *Methylocystis parvus* OBBP, a Type II strain and *Methylobacter marinus* A45, a Type I strain. *Methylobacter albus* BG8 DNA was used as a control. Hybridization to the DNA from the other methanotrophs was observed in all cases with all three probes, although it was not as strong as that with *Methylobacter albus* BG8 (Fig. 7). In the case of *Methylococcus capsulatus* Bath, the major bands that hybridized to each of the three probes were of different sizes. However, for the other two strains, the *mxmCK* probe hybridized to two bands that were the same size as the bands detected by the *mxmA* and *mxmL* probes, respectively. In the case of *Methylobacter marinus* A45, other bands were also detected by the *mxmCK* probe. These data suggest that in *Methylocystis parvus* OBBP and *Methylobacter marinus* A45, the *mxmCK* region lies between the *mxmA*

and *mxmL* regions, just as it does in *Methylobacter albus* BG8 and *Methylobacterium extorquens* AM1.

DISCUSSION

The methanol oxidation (Mox) system in the facultative methanol utilizers appears to be complex, requiring a large number of genes to carry out the single biochemical step of converting methanol to formaldehyde (Anderson & Lidstrom, 1988; Lidstrom, 1990; Machlin *et al.*, 1988; Nunn & Lidstrom, 1986a; Lidstrom *et al.*, 1994). These functions include the structural genes for the MDH and its electron acceptor (*cytc_L*), genes involved in synthesis of the cofactor PQQ, regulatory genes, and genes necessary for assembly (Anderson & Lidstrom, 1988; Lidstrom, 1990; Nunn & Lidstrom, 1986a). Previous work has shown that the MxvF polypeptides from a variety of methylophs show similarity (Anthony, 1986), and that the genes encoding the large subunit of the MDH are also conserved (Anderson *et al.*, 1990; Harms *et al.*, 1987; Machlin & Hanson, 1988; Stephens *et al.*, 1988).

The results presented here suggest that some of the other genes in the Mox system are also conserved among methane and methanol utilizers, based on specific hybridization of genomic fragments to *mxmA*, *K* and *LD* probes. These *mxm* genes are involved in producing a functional MDH holoenzyme, consisting of the MDH apoprotein, its cofactor PQQ, and calcium (Richardson & Anthony, 1992), and therefore might be expected to be present in other Mox systems. The hybridization between *Methylobacterium extorquens* AM1 *mxm* genes and methanotrophic DNA was strongest for the *mxmLD* probe and much weaker for the *mxmA* and *mxmK* probes. In the case of *Methylobacter albus* BG8, an 8.5 kb *Hind*III fragment was isolated that hybridized to all three *Methylobacterium extorquens* AM1 probes, and each probe hybridized to a specific set of subfragments. In this case, the hybridization was strongest to the *mxmLD* probe and weaker to the *mxmK* probe, as with the genomic digests, but it was surprisingly strong to the *mxmA* probe. It is not clear why the *mxmA* probe hybridized better to the cloned fragment than the genomic digests, while the *mxmK* probe hybridized weakly in both cases. In side-by-side hybridizations under the same conditions, the same results were always obtained. Fragments containing the *mxmA*, *CK* and *L* regions of *Methylobacter albus* BG8 detected specific bands of genomic DNA from other methanotrophs, and in the case of the Type II strain *Methylocystis parvus* OBBP and the Type I strain *Methylobacter marinus* A45, the hybridization pattern suggested that the order of the genes was the same as in *Methylobacter albus* BG8 and *Methylobacterium extorquens* AM1.

Expression studies of the cloned *Methylobacter albus* BG8 fragment showed that the region of hybridization to the *Methylobacterium extorquens* AM1 *mxmA* *AKL* genes encoded five polypeptides, and a sixth was encoded downstream of these in a region that did not show significant hybridization to the *mxmA* *AKL* probes. Recent data have shown that the *mxmA* *AKL* region of *Methylobacterium extorquens* AM1 contains five genes, *mxmA* *ACKLD*, with calculated

molecular masses of 33·7/31·8, 37·5, 23, 35·6/32·6 and 18·5/16·6 kDa, respectively, with the pairs representing the mature and processed forms of putative periplasmic and membrane polypeptides (Morris *et al.*, 1995). However, attempts to express these genes in an *E. coli* expression system were not successful, with the exception of *mxsD*. In our study, these five genes were all expressed in *E. coli*. The region that hybridized to the *mxsA* probe expressed a polypeptide of 33/32 kDa, the region that hybridized to the *mxsK* probe and also hybridized weakly to the *mxsLD* probe expressed polypeptides of 26·5 and 20 kDa, and the region that hybridized well to the *mxsLD* probe expressed a polypeptide of 38·5 kDa. In addition, preliminary sequencing across the N₁ *Nru*I site revealed the presence of a gene with identity to *mxsC* and preliminary sequencing across the E₂ *Eco*RI site revealed the presence of a gene with identity to *mxsL*. These data suggest that the 33/32 kDa polypeptide corresponds to *mxsA*, the 38 kDa polypeptide corresponds to *mxsC*, the 26·5 kDa polypeptide corresponds to *mxsK*, the 20 kDa polypeptide corresponds to *mxsD* and the 38·5 kDa polypeptide corresponds to *mxsL*. Fig. 6 shows the assumed sizes of these genes, based on the expression data. The region involved in both hybridization and expression is just large enough to encode all of these polypeptides. The identity of the 23·5 kDa polypeptide expressed in the region downstream of *mxsL* is not known. These data suggest that the relative order of *mxsL* and *D* is reversed in *Methylobacter albus* BG8 compared to *Methylobacterium extorquens* AM1. It is not yet known whether *mxsD* has a function in calcium acquisition by MDH. Our data do not allow us to determine the order of *mxsK* and *D*.

Despite the evidence outlined above that *mxsA*, *K* and *L* are present in *Methylobacter albus* BG8, we were unable to demonstrate functional complementation of *Methylobacterium extorquens* AM1 *MxsA*, *K* and *L* mutants. The lack of heterologous complementation could be due to lack of expression, insufficient functional similarity, or both. The T7 expression data suggest that one of the fragments tested (H₁–H₂), contains complete *mxsA*, *K* and *L* and two of them (E₁–E₂ and B₁–B₂) contain complete *mxsA* and *mxsK* genes. Therefore, these clones should have complemented the *Methylobacterium extorquens* AM1 mutants. However, earlier studies indicated that a *Methylobacterium extorquens* AM1 *MxsF* mutant could only be complemented by the *Methylobacter albus* BG8 *mxsF* when small subclones (less than 5 kb) were used (Stephens *et al.*, 1988). Larger subclones containing *mxsF* did not complement, presumably reflecting difficulties in expression. *MxsA* and *K* mutants of *Methylobacterium organophilum* XX and *Methylobacterium extorquens* AM1 have been complemented with DNA from a Type II methanotroph, *Methylosinus methanica* (Bastien *et al.*, 1989). Therefore, at least in some instances functional conservation is sufficient for complementation. Further work will be necessary to determine whether expression occurs in *Methylobacterium extorquens* AM1 but the gene products are not functional, or whether expression does not occur.

In *Methylobacterium extorquens* AM1, *mxsF* (encoding the

60 kDa subunit of the MDH) is located in a gene cluster adjacent to the *mxsA*, *K* and *L* genes in the order F–J–G–I–R–S–A–C–K–L–D (Lidstrom, 1990; Morris *et al.*, 1995). The distance between *mxsF* and *mxsA* in *Methylobacterium extorquens* AM1 is approximately 5 kb (Lidstrom, 1990). However, an analysis of the restriction fragments for the *Methylobacter albus* BG8 *mxsF* and *mxsA*, *K* and *L* clones shows no overlap on either side, and so these genes must be separated by at least 6 kb (Stephens *et al.*, 1988).

Genetic studies of the Mox system in obligate methanotrophs are difficult, as mutants in this system are lethal and conditional mutants are difficult to obtain. Therefore, we have approached these studies with surrogate genetics, using metabolically related organisms as model systems. The cloning of two sets of Mox genes from *Methylobacter albus* BG8 (*mxsF* and the *mxsA*, *C*, *K*, *D* and *L* genes) now opens the way to studies of coordinated gene expression in this important group of bacteria.

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