The *mxaAKL* genes of *Methylobacter albus* BG8

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**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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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 mxaA, K and L, which are located immediately 3' to a cluster of mxa genes (mxaFJGIRS) in *Methylobacterium extorquens* AM1 (Nunn & Lidstrom, 1986a; Lidstrom et al., 1994; Morris et al., 1995). The mxaA, 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 mxaA, 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 (mxaC and mxaD) 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 mxaAKL in *Methylobacterium extorquens* AM1 are part of a large gene cluster that includes (in order) mxaFJGIRSACKLD.

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 mxaA, K and L genes from a Type I methanotroph. A gene probe derived from cloned *Methylobacterium extorquens* AM1 DNA containing mxaL was used to identify specific hybridizing fragments in genomic digests of DNA from a variety of obligate methanotrophs, and to clone mxaA, 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 mxaACKLD of *Methylobacterium extorquens* AM1. Functional complementation of *Methylobacterium extorquens* AM1 MxaA, 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 Hardest 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; 125 μg tetracycline ml⁻¹; 10 μg rifamycin 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 *Methyllobacter 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 *Methylobacter extorquens* AM1 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* AM1 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-Horowicz & 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 10 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

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<tr>
<th>Strains</th>
<th>Relevant traits</th>
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<tr>
<td>E. coli DH5α</td>
<td>r&lt;sup&gt;-&lt;/sup&gt; m&lt;sup&gt;+&lt;/sup&gt; recA1 galD::lacZΔM15 (lacZYA-argF)U169 hsdR</td>
<td>BRL</td>
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<td>HB101</td>
<td>r&lt;sup&gt;-&lt;/sup&gt; m&lt;sup&gt;-&lt;/sup&gt; hsdS recA13 ara-14 supE lacY mcrB galK proA xyl-5 mit-1 lev-1 rpsL</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
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<td>AM1 rif</td>
<td>Rif&lt;sup&gt;R&lt;/sup&gt;-derivative</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
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<td>AM1 PG1</td>
<td>MxaA mutant of AM1, rif&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>MxaK mutant of AM1, rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
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<td>MxaL mutant of AM1, rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
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<td><strong>Methane utilizers</strong></td>
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<td><strong>Type X</strong></td>
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<td>Tc&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt; IncP1 cosmid</td>
<td>Knauf &amp; Nester (1982)</td>
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<td>pRK310</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt; lacP0Z&lt;sup&gt;R&lt;/sup&gt; IncP1</td>
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<td>Km&lt;sup&gt;R&lt;/sup&gt;-mobilizing plasmid</td>
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<td>pCT2</td>
<td>pRK310 (0.9 kb SalI-EcoRI); mxaK</td>
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<td>pT76 (4.0 kb BamHI-BamHI)</td>
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<td>pCT157</td>
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Rstriction 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.
Protein expression. Protein expression from cloned genes was accomplished using the T7 promoter vectors pT7-5 and pT7-6 and plasmid pGSP1-2 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 mxaA, K and L genes

In order to determine whether similarity exists among the mxaA, 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 PstI–BamHI fragment containing mxaA, a 0-9 kb SacII–EcoRI fragment containing mxaK, and a 1-8 kb SalI–SalI fragment containing mxaL (Fig. 1a; Nunn & Lidstrom, 1986a). The latter fragment is now known to contain mxaD also (Morris et al., 1995).

Each of these isolated fragments was used to probe EcoRI 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 conditions were tested, but conditions allowing 30–37% base pair mismatch (see Methods) produced the clearest results. With the mxaA and mxaK 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 mxaLD 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. strain; OB3b; 3, Methylocystis parvus OB8B; 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.

Cloning of the mxa genes from Methylobacter albus BG8

The data presented above suggest that significant similarity of the mxaLD genes occurs in diverse methanotrophs and some conservation of sequences may also be present in mxaA and K genes. In order to determine whether the hybridization observed was due to the presence of true mxaA, K and L homologues, we cloned the corresponding DNA fragments from one of the methanotrophs. Methylobacter albus BG8 was chosen, since the mxaLD probe showed good hybridization to Methylobacter albus BG8 genomic DNA (Fig. 2), and a genomic clone bank (a HindIII 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 (data not shown).
Each of these clones contained an identical set of HindIII fragments of approximately 8.5, 7.0, 3.4, 3.2, 1.4 and 1.3 kb in size. The mxaLD 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 EcoRI (E1-E2) fragment as identified in the initial genomic blots (Fig. 2). When blots of genomic EcoRI, HindIII and BamHI digests were tested with the 4 kb BamHI Methylobacter albus BG8 fragment (B1-B2 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 mxaAKL regions**

Each of the Methylobacterium extorquens AM1 mxa probes was used for hybridization to restriction digests of the 8.5 kb HindIII fragment or an appropriate subclone in order to delineate the position of each of the mxa genes in Methylobacter albus BG8. The mxaLD probe hybridized most strongly to a 0.8 kb PstI–BamHI (P1–B2) fragment and more weakly to the adjacent 3.3 kb BamHI–PstI (B1–P3) fragment (Figs 1 and 4a). The cloned 5.9 kb EcoRI and 8.5 kb HindIII fragments were probed with the Methylobacterium extorquens AM1 mxaA and mxaK probes, respectively, and in both cases, the strongest region of hybridization was to the 3.3 kb BamHI–PstI (B1–P3) and the 4.0 kb PstI–PstI (P1–P3) fragments located to the left of the mxaLD-hybridizing region (Figs 1 and 4b, c). The mxaA probe also hybridized weakly to the adjacent 1.2 kb SalI–BamHI region (S1–B2), and the mxaK probe hybridized more strongly to the 3.4 kb NruI–NruI (N1–N4) fragment than the adjacent 4 kb HindIII–NruI (H1–N4) fragment. These data suggested that the region similar to mxaLD is located to the right of the region similar to mxaA and mxaK, and the region similar to mxaK is...
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 DH5a 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, pBMX854ABN; 8, pBMX860ABN. (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 mxaA, as shown in Fig. 1b. No significant hybridization was observed with the mxaA or mxaK probe to the 0.8 kb PstI–BamHI (P$_3$–B$_2$) fragment that hybridized to the mxaLD probe. As shown in Fig. 4(c), hybridization of the mxaK 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 mxaA, 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 40 kb BamHI (B$_1$–B$_2$) fragment containing the region that hybridized most strongly to the mxaA, 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 BamHI (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 mxaAKL 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 BamHI–NraI (B$_1$–N$_1$) fragment, which is the region that hybridized most strongly to the mxaA probe. The 38 kDa polypeptide was only expressed from the plasmids containing DNA across the N$_2$, NraI 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.
upstream. Preliminary sequencing of the region around the N₇, NrlI site revealed a partial ORF with 39% identity to the Methylobacterium extorquens AM1 mxaC across a 118 amino acid overlap (A. Springer & M. Lidstrom, unpublished data), suggesting that the 38 kDa polypeptide corresponded to MxaC. The 26.5 and 20 kDa polypeptides were expressed from all clones containing the 1.6 kb NrlI–PstI (N₁–P₁) fragment, just downstream of the region encoding the 33/32 kDa polypeptides. This was the region that hybridized most strongly to the mxaK probe, but it also hybridized to the mxaLD probe. The region from the B₁ site to the P₃ 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 NrlI–BamHI (N₁–B₄) fragment that includes DNA downstream of the region encoding the 26.5 and 20 kDa polypeptides. This downstream region hybridized most strongly to the mxaLD 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 (E₂–B₄) or 0.8 kb PstI–BamHI (P₃–B₄) fragments. This indicates that none of these polypeptides are wholly encoded upstream of the EcoRI site denoted E₂ 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 P₃ in Fig. 6. Preliminary sequencing upstream of the E₂ site revealed a partial ORF that showed 31% identity to mxaL of Methylobacterium extorquens AM1 across 39 amino acids (A. Springer & M. Lidstrom, unpublished data). In Methylobacterium extorquens AM1, mxaL is predicted to encode a 37 kDa polypeptide, suggesting that this gene might extend across the B₄ site. In order to test this hypothesis, two other subclones were used for expression that include DNA past that site to the HindIII site (H₂) (Figs 5b and 6). In both cases, none of the polypeptides observed from the B₁–B₂ clones were present. For the P₃–H₂ subclone, two new polypeptides of 38.5 and 23.5 kDa were present, while in the smaller B₃–H₂ 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 mxaL, that crosses the B₃ 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 MxaA, K and L mutants

The results presented above suggest that Methylobacter albus BG8 contains genes equivalent to the mxaA, K and L genes of Methylobacterium extorquens AM1. Having cloned and localized these mxa genes and also shown their expression in E. coli, we attempted to complement the MxaA, 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 (H₁–H₂, E₁–E₂, B₁–B₂), cloned in both orientations, the PstI–HindIII (P₃–H₂) fragment cloned right to left with respect to the lac promoter in pRK310 and the PstI–EcoRI (P₃–E₂) 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 MxaA, 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 mxaA, CK and L probes to DNA from methanotrophs. Probes: (a) mxaA; (b) mxaCK; (c) mxaL. Lanes: 1, Methylobacter albus BG8; 2, Methylococcus capsulatus Bath; 3, Methylocystis parvus OBPP; 4, Methylobacter marinus A45. Sizes of standards are shown on the right. Chromosomal DNA was digested with BstYI.

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 shown on the right. Chromosomal DNA was digested with OBBP; 4, mxaA, CK negative controls with no vector, confirming that plasmid transfer had occurred.

Hybridization to methanotroph DNA

Three subclones of the Methylobacter albus BG8 BamH1 fragment containing the putative mxaA, 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 mxaL probe. The three subclones were B3–N1, which should contain mainly mxaA, N2–P2, which should contain mxaK and the major portion of mxaC, and P3–B2, which should be specific to mxaL (Fig. 1). The strains tested were Methylococcus capsulatus Bath, a Type X strain, Methylocystis parvus OBPP, 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 mxaCK probe hybridized to two bands that were the same size as the bands detected by the mxaA and mxaL probes, respectively. In the case of Methylobacter marinus A45, other bands were also detected by the mxaCK probe. These data suggest that in Methylocystis parvus OBPP and Methylobacter marinus A45, the mxaCK region lies between the mxaA and mxaL 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 (cyto), 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 MxaF polypeptides from a variety of methylo trophs 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 mxaA, K and L probes. These mxa 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 Methylo bacterium extorquens AM1 mxa genes and methanotrophic DNA was strongest for the mxaLD probe and much weaker for the mxaA and mxaK probes. In the case of Methylobacter albus BG8, an 8.5 kb HindIII 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 mxaLD probe and weaker to the mxaK probe, as with the genomic digests, but it was surprisingly strong to the mxaA probe. It is not clear why the mxaA probe hybridized better to the cloned fragment than the genomic digest, while the mxaK probe hybridized weakly in both cases. In side-by-side hybridizations under the same conditions, the same results were always obtained. Fragments containing the mxaA, 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 OBPP 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 mxaAKL genes encoded five polypeptides, and a sixth was encoded downstream of these in a region that did not show significant hybridization to the mxaAKL probes. Recent data have shown that the mxaKL region of Methylobacterium extorquens AM1 contains five genes, mxaACKLD, 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 mxaD. In our study, these five genes were all expressed in E. coli. The region that hybridized to the mxaA probe expressed a polypeptide of 33/32 kDa, the region that hybridized to the mxaK probe and also hybridized weakly to the mxaLD probe expressed polypeptides of 26.5 and 20 kDa, and the region that hybridized well to the mxaLD probe expressed a polypeptide of 38.5 kDa. In addition, preliminary sequencing across the N1 NruI site revealed the presence of a gene with identity to mxaC and preliminary sequencing across the E9 EcoRI site revealed the presence of a gene with identity to mxaL. These data suggest that the 33/32 kDa polypeptide corresponds to mxaA, the 38 kDa polypeptide corresponds to mxaC, the 26.5 kDa polypeptide corresponds to mxaK, the 20 kDa polypeptide corresponds to mxaD and the 38.5 kDa polypeptide corresponds to mxaL. 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 mxaL is not known. These data suggest that the relative order of mxaL and D is reversed in Methylobacterium BG8 compared to Methylobacterium extorquens AM1. It is not yet known whether mxaD has a function in calcium acquisition by MDH. Our data do not allow us to determine the order of mxaK and D.

Despite the evidence outlined above that mxaA, K and L are present in Methylobacterium albiius BG8, we were unable to demonstrate functional complementation of Methylobacterium extorquens AM1 MxaA, 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 mxaA, K and L and two of them (E1-E2 and B1-B2) contain complete mxaA and mxaK genes. Therefore, these clones should have complemented the Methylobacterium extorquens AM1 mutants. However, earlier studies indicated that a Methylobacterium extorquens AM1 MxaF mutant could only be complemented by the Methylobacterium albiius BG8 mxaF when small subclones (less than 5 kb) were used (Stephens et al., 1988). Larger subclones containing mxaF did not complement, presumably reflecting difficulties in expression. MxaA 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, mxaF (encoding the 60 kDa subunit of the MDH) is located in a gene cluster adjacent to the mxaA, 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 mxaF and mxaA in Methylobacterium extorquens AM1 is approximately 5 kb (Lidstrom, 1990). However, an analysis of the restriction fragments for the Methylobacterium BG8 mxaF and mxaA, 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 Methylobacterium albiius BG8 (mxaF and the mxaA, 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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