Methanol oxidation mutants in *Methylobacterium extorquens* AM1: identification of new genetic complementation groups

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Two-hundred-and-eight new *Methylobacterium extorquens* AM1 methanol oxidation (Mox) mutants were isolated and placed into complementation groups. Complementation analyses identified new Mox groups in the Mxb and Mxc loci and at a new locus, Mxd. Thirty-seven mutants at the Mxb locus were divided into MxbM and MxbD complementation groups on the basis of their complementation pattern. Twenty-nine mutants at the Mxc locus fell into three complementation groups, MxcB, MxcQ and MxcE. The direction of transcription for genes at this locus could be inferred from the subclones. Eighteen of the new mutants were not complemented by previously isolated *M. extorquens* AM1 clones but were complemented by two new overlapping clones. This locus was called Mxd and the mutants fell into two complementation groups, MxdR and MxdS. Immunoblots from all these mutant classes showed that all of the Mxb and Mxc strains had substantially reduced levels of MxaF (large subunit of methanol dehydrogenase) and cytochrome *c* compared to the wild-type. These mutants, particularly the Mxb mutants, also had elevated levels of cytochrome *c*553. These results are consistent with a role for the MxbMD and MxcBQE complementation groups in the regulation of expression of *mxaF*. The MxdR and MxdS mutants had normal levels of MxaF and both c-type cytochromes.

Keywords: *Methylobacterium extorquens*, methylotrophs, methanol oxidation, *mox* genes

INTRODUCTION

Methylotrophic bacteria grow on one carbon compounds as their sole source of carbon and energy. Gram-negative methylotrophs that grow on methane or methanol use the quinoprotein methanol dehydrogenase (MDH) to oxidize methanol to formaldehyde (Lidstrom, 1991). Formaldehyde can then be further oxidized or it can be assimilated into cell carbon (Lidstrom, 1991). Structural studies indicate that MDH is an αβ₄ tetramer that contains calcium and the prosthetic group pyrroloquinoline quinone (PQQ) at the active site (Xia *et al.*, 1992; White *et al.*, 1993; Richardson & Anthony, 1992; Anthony, 1986; Anthony *et al.*, 1994; Ghosh *et al.*, 1995).

Genetic analyses have been used to study methanol oxidation in the facultative methanol-utilizing bacterium *Methylobacterium extorquens* AM1. A positive selection for Mox (methanol oxidation) mutants exists that takes advantage of the broad specificity of MDH. Allyl alcohol can be oxidized by MDH but the product, allyl aldehyde, is toxic to the cell. Therefore, while *M. extorquens* AM1 normally cannot grow in the presence of allyl alcohol, mutagenized cells defective in the Mox system can grow. This selection has permitted the isolation of a number of Mox mutants, and complementation analyses of these mutants has led to the identification of several Mox genes. The gene *mxaF* (formerly *moxF*, see Lidstrom *et al.*, 1994) encodes the 66 kDa MDH large subunit, *mxaI* (formerly *moxI*) encodes the 9 kDa MDH small subunit and *mxaG*...
(formerly moxG) encodes the cytochrome $\epsilon_6$ electron acceptor for MDH (Anderson & Lidstrom, 1988; Nunn & Lidstrom, 1986a, b). These three genes are clustered together with a fourth gene, mxaI, of unknown function. Immediately downstream of mxaI are two more genes, mxaR and S, which have been identified in Paracoccus denitrificans (van Spanning et al., 1991). Sequence comparisons suggest that these genes are also present in M. extorquens AM1 (van Spanning et al., 1991; C. J. Morris & M. E. Lidstrom, unpublished), but their function is unknown. Immediately downstream of the MDH complex (Richardson et al., 1992), a collection of five genes, mxaACKLD, the first four of which are required for the proper insertion of calcium into the MDH complex (Richardson & Anthony, 1992; C. J. Morris & M. E. Lidstrom, unpublished). The function of mxaD is unknown.

Further studies in M. extorquens AM1, Methylobacterium organophilum XX and other methylotrophs indicate that a number of additional genes are required for oxidation of methanol (Bastien et al., 1989; Laufer & Lidstrom, 1992). There are seven pqq genes required for biosynthesis of PQQ (Morris et al., 1994). In addition, there are at least six complementation groups (MxaB, MxcQ, MxbMDN, formerly MoxB, Q, E, M, D and N, respectively) required for transcription of mxaF and other Mox genes (Nunn & Lidstrom, 1986b; Lidstrom, 1992; Morris & Lidstrom, 1992a; Xu et al., 1993). One characteristic of the MoxD mutants is the appearance of a novel $\epsilon$-type cytochrome, cytochrome $\epsilon$-553 (Nunn & Lidstrom, 1986b; Day et al., 1990). This cytochrome has been purified, but its function in M. extorquens AM1 is unknown (Anthony, 1992).

In an attempt to identify more Mox complementation groups, a collection of 214 Mox mutants has been generated in M. extorquens AM1 (Morris et al., 1994). Seventy-two mutants from this collection are required for PQQ biosynthesis and their characterization has already been described (Morris et al., 1994). The characterization of 14 mutants in the Mxa cluster, including those required for insertion of calcium, is described elsewhere (C. J. Morris & M. E. Lidstrom, unpublished). This study describes the characterization of new mutants at the Mxb and Mxc loci, and the identification of two new Mox complementation groups, MxdR and MxdS.

**METHODS**

**Bacterial strains and plasmids.** All Mox mutants were derived from rifamycin-resistant M. extorquens AM1 (Nunn & Lidstrom, 1986a), except for the M. organophilum XX strains SM2 (MxbN mutant, formerly group VI-B; Bastien et al., 1989) and SM3 (MxbD mutant, formerly group VI-C; Bastien et al., 1989) that were kindly provided by R. S. Hanson (Minnesota). The following M. extorquens AM1 strains were used for immunoblot and complementation analyses: MxbM, 7-20; MxbD-I, 7-19; MxbD-II, 7-14; MxcB, 7-25; MxcQ, 8-10; MxcE, 7-12; MxdR, EMM53; MxdS, EMM52. Escherichia coli DH5a was used for DNA manipulation (BRL). Plasmids used in complementation analyses were clones in pRK2073 (Sm$^\beta$, Figurski & Helinski, 1979) and pRK2013 (Km$^\beta$, Figurski & Helinski, 1979). The transposon TnphoA was inserted into pDN202 using P22-Mud::TnphoA (Sanderson & Roth, 1988) by the method of Hughes & Roth (1988) and as described by Morris et al. (1994). Isolation of complementing clones for Mxd mutants was performed using the technique described by Nunn & Lidstrom (1986a).

**Media and growth conditions.** M. extorquens AM1 strains were grown at 30°C on minimal medium described previously (Fulton et al., 1984) containing 0.2% (v/v) methanol, 0.2% (w/v) methylamine, 0.2% (w/v) succinate, or 0.5% methanol plus 0.2% methylamine. Antibiotics were added at the following final concentrations (mg l$^{-1}$): tetracycline, 12.5; kanamycin, 25; streptomycin, 10. Chemicals were obtained from Sigma. Nutrient agar and Bacto-agar were obtained from Difco.

**Isolation of Mox mutants.** Mox$^-$ mutants of M. extorquens AM1 were isolated using the allyl alcohol selection procedure (Nunn & Lidstrom, 1986a). Rifamycin-resistant M. extorquens AM1 was mutagenized with EMS as described by Morris et al. (1994).

**Bacterial matings, complementation analysis and recombination rescue.** Triparental matings between E. coli strains and M. extorquens AM1 strains containing pVK100 derivatives were performed overnight at 30°C on nutrient agar plates. Exconjugates were then diluted and plated on minimal medium containing methanol, succinate or methylamine, the appropriate antibiotic solution, and rifamycin to select against E. coli. The number of colonies appearing on minimal agar containing methanol was compared to the number of colonies appearing on minimal agar containing methylamine or succinate. Positive complementation was scored when the number of colonies was significantly more than that seen for spontaneous reversion of the mutant phenotype. This was scored as recombination rescue. This phenomenon is believed to result from recombination between the cloned fragment and the mutated region on the chromosome of the mutant strain. Such an event would indicate that the cloned fragment contains at least part of the gene defective in the mutant strain in question. In all experiments, matings using vector (pVK100 or pRK310) alone were used as negative controls.

**Preparation of cell extracts and immunoblots.** Crude extracts of M. extorquens AM1 strains grown to late exponential phase on 0.5% methanol plus 0.2% methylamine were made using a French Press as described by Chistoserdova & Lidstrom (1992). Protein concentrations were estimated spectrophotometrically (Whitaker & Granum, 1980). Approximately 15 µg protein from each crude extract was loaded onto SDS-polyacrylamide gels and visualized using Coomassie blue R or electrophoretically transferred onto nitrocellulose for immunoblot analysis. Incubation of gels with antisera was carried out as described previously (Nunn & Lidstrom, 1986a). The results were visualized using alkaline phosphatase colour development reagents (Bio-Rad). Antibody to MDH has been described previously (Lidstrom, 1986b; Anthony, 1992). Anti-cytochrome $\epsilon$ was obtained from D. Nunn (Illinois); anti-cytochrome $\epsilon$-553 was obtained from C. Anthony (Southampton).

**DNA manipulation.** Preparation of subclones was performed using E. coli DH5a following the methods of Sambrook et al. (1989) and as described previously (Morris et al., 1994). DNA fragments for subcloning were purified using GeneClean (Bio101). Enzymes were obtained from New England Biolabs and Boehringer-Mannheim.
RESULTS

New Mox mutants

Two-hundred-and-fourteen new Mox mutants of \textit{M. extorphens} AM1 were isolated by allyl alcohol selection. These were tested for complementation by cosmid clones of \textit{M. extorphens} AM1 that had been shown to complement other Mox mutants. The clones, listed in Table 1, contain HindIII fragments of 8–20 kb and are described by Nunn & Lidstrom (1986a). Of the 214 Mox mutants, six from the original collection were lost, leaving 208 to be studied further. One-hundred-and-ninety of the Mox mutants were complemented by known clones, as shown in Table 1. The remaining 18 mutants, as shown below, make up a new locus designated Mxd. All of the 208 mutants were subdivided further into smaller complementation groups.

The remaining nine were MxaG or MxaI mutants since they were not complemented by a clone complementing only MxaF and MxaJ (data not shown). Since these loci have already been extensively studied (Anderson & Lidstrom, 1988; Anderson \textit{et al.}, 1990; Nunn & Lidstrom, 1986a, b; Morris & Lidstrom, 1992a), these new Mxa mutants were not characterized further.

The Mxb complementation group

Thirty-seven mutants were mapped by complementation to the HindIII-D clone but were not in the Pqq complementation groups described by Morris \textit{et al.} (1994). Thirty-six of these mutants were also complemented by p1130D, which contains a 6.5 kb HindIII–PstI fragment (Fig. 1). The Mxb mutants were further divided into three classes, as listed in Table 1 and as shown in Fig. 1. The MxbM complementation group was complemented by p1130D-HB, containing 4 kb from the left-hand side of the p1130D fragment, but not by p1130D-HBg2 or p1130D-PBg. Twenty-one of the new mutants showed this complementation pattern. This pattern indicates that mxb\textit{M} must lie across the second BglI site in p1130D (Fig. 1). Mutants in the MxbD complementation group were not complemented by p1130D-HB but were comple-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Complementing fragment} & \textbf{Group} & \textbf{No. of mutants} & \textbf{Reference} \\
\hline
HINDIII-AB & MxaA & 4 & C. J. Morris & M. E. Lidstrom, unpublished \\
 & MxaC & 5 & C. J. Morris & M. E. Lidstrom, unpublished \\
 & MxaK & 3 & C. J. Morris & M. E. Lidstrom, unpublished \\
 & MxaL & 1 & C. J. Morris & M. E. Lidstrom, unpublished \\
 & MxaB & 2 & C. J. Morris & M. E. Lidstrom, unpublished \\
HINDIII-D & PqqG & 5 & Morris \textit{et al.} (1994) \\
 & MxbM & 21 & This study \\
 & MxbD I & 12 & This study \\
 & MxbD II & 3 & This study \\
 & MxbMD' & 1 & This study \\
HINDIII-E & MxbE & 7 & This study \\
 & MxcQ & 8 & This study \\
 & MxcE I & 6 & This study \\
 & MxcE II & 6 & This study \\
 & MxcQE* & 2 & This study \\
HINDIII-FG & MxaF & 29 & This study \\
 & MxaGI & 9 & This study \\
Other & MxdR & 5 & This study \\
 & MxdS & 13 & This study \\
\hline
\textbf{Total} & & 208 & \\
\hline
\end{tabular}
\caption{Complementation groups of the 208 new Mox mutants}
\end{table}

The numbers of mutants in each complementation group determined so far are shown. The complementation groups for which no mutants were found in this study, i.e. MxaJRSD, PqqB and PqqD are not shown.
Fig. 1. Restriction map and complementation analysis of MxbM and MxbD mutants of *M. extorquens* AM1, using derivatives of the HINDIII-D clone. The position of *ppqdGC* is indicated with boxes (Morris et al., 1994). XX is used to indicate the *M. organophilum* XX MxbD and MxbN strains. These strains are not expected to show recombinational rescue with *M. extorquens* AM1 complementing clones. Arrows indicate the direction of the *lacZ* promoter on the pRK310 vector and of the *ppqd* promoter at top of figure. B, BamHI; G, BglII; H, HindIII; P, PstI; S, SalI. Shaded lines indicate the approximate region covered by the respective complementation group (M or D). ‘r’ in the complementation table indicates recombinational rescue (see Methods); ND indicates no data available.

<table>
<thead>
<tr>
<th>Figure 1 Data</th>
<th>Complementation</th>
<th>MxbM</th>
<th>MxbD</th>
<th>XXMxbD</th>
<th>XXMxbN</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>p1130D-HB</td>
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<td>r</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>p1130D-HBg2</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
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<tr>
<td>p1130D-PBg</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>pALS11</td>
<td>-</td>
<td>-</td>
<td>r</td>
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</table>

Fig. 2. Restriction map and complementation analysis of MxcB, MxcQ and MxcE mutants of *M. extorquens* AM1, using derivatives of the HINDIII-E clone, pDN202. Small arrows indicate the direction of the *lacZ* promoter on the prK310 vector. B, BamHI; E, EcoRI; H, HindIII; Sp, SphI. The filled triangle represents a TnphoA insertion. Shaded lines indicate the approximate region covered by the respective complementation group (B, Q or E), with arrowheads indicating the inferred direction of transcription. ‘r’ in the complementation table indicates recombinational rescue (see Methods).

<table>
<thead>
<tr>
<th>Figure 2 Data</th>
<th>Complementation</th>
<th>MxbB</th>
<th>MxcQ</th>
<th>MxcE</th>
<th>QE*</th>
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<td>pDN202</td>
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<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pDN202::TnphoA-Q1</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pALS2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>pALS3</td>
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</tr>
<tr>
<td>pHHC21</td>
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<td>r</td>
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<td>r</td>
</tr>
<tr>
<td>pHHC22</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
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</tbody>
</table>

One MxbMD mutant, EMS63, showed recombinational rescue with p1130D, and p1130D-PBg, but was not complemented by any clone (Table 1; data not shown in Fig. 1). This phenotype was referred to as ‘MxbMD⁺’, the superscript indicating recombinational rescue. Such a phenotype may indicate that there is a gain-of-function mutation in this strain. This strain was not characterized further.

*M. organophilum* XX MxbD and MxbN mutants had complementation patterns similar to the MxbD mutants...
New methanol oxidation mutants in *M. extorquens* AM1

The Mxd complementation group

Twenty-nine mutants were complemented by the HINDIII-E clone (pDN202) and by pALS2 (Fig. 2). Eight of these mutants were not complemented by a clone containing a TnphoA insertion in pDN202 (pDN202-TnphoA-Q1; Fig. 2). These mutants were designated MxQ. The MxQ mutants were mapped by complementation to lie within the 1.8 kb HindIII-SphI fragment in pHHC21. The remaining 19 Mx mutants that were complemented by pDN202-TnphoA-Q1 fell into two groups: those that were complemented by pHHC21, and those that were not (Fig. 2). The latter group was designated MxE, and included 12 of the new mutants as well as the original MxE mutants (Nunn & Lidstrom, 1986a). Six MxE mutants (in addition to the original MxE mutants) could be recombinationally rescued by pHHC21 (Class I) and six could be recombinationally rescued by the adjoining SphI fragment found in pHHC11 (Class II). These results suggest that *mxe* lies across this SphI site. The group that was complemented by both pHHC21 and pDN202-TnphoA-Q1 contained seven mutants, and has been designated MxB.

The fragments in pHHC11 and pHHC21 were also cloned in the opposite orientation relative to the lacZ promoter of the *pRK310* vector. These plasmids were called pHHC12 and pHHC22, respectively. The clone pHHC12 did not complement any of the mutants, but could recombinationally rescue all of the Class II MxE mutants (Fig. 2). The clone pHHC22 rescued all the MxQ, MxB and Class I MxE mutants, but did not complement any mutants (Fig. 2). These results suggest that the direction of transcription of these genes is the same as the direction of the lacZ promoter in pHHC21.

Two of the mutants complemented by pALS2 were not complemented by pDN202-TnphoA-Q1 or by pHHC21. These mutants are therefore both MxQ" and MxE", and have been called ‘MxQE*’. The implications of this phenotype are discussed below.

The Mxb complementation group

Eighteen mutants were not complemented by known clones. The HindIII clone bank described by Nunn & Lidstrom (1986a) was used to isolate two complementing plasmids containing inserts of 18.2 and 20.6 kb. These plasmids were restriction-mapped and shown to have extensive overlap (Fig. 3). This indicates that all 18
Fig. 5. (a) Immunoblot of extracts of Mox mutants probed with anti-cytochrome cL antibody. Extracts were run on polyacrylamide gels as described in the legend to Fig. 4. (b) Immunoblot of extracts of Mox mutants probed with anti-cytochrome c-553 antibody. Std indicates protein molecular mass standards (low range, Bio-Rad).

Phenotypic characterization of complementation groups at Mxb, Mxc and Mxd loci

Strains from each of the identified Mxb, Mxc and Mxd complementation groups were tested for the presence of MDH large subunit (MxaF), cytochrome cL and cytochrome c-553 using immunoblots. For these experiments, crude extracts were prepared from strains grown in the presence of methanol plus methylamine, conditions that should induce Mox functions in Mox mutants (Nunn & Lidstrom, 1986b). The results are shown in Figs 4 and 5. All the Mxb and Mxc mutants tested showed reduced levels of MxaF and cytochrome cL compared to the wild-type. In addition, all of the Mxb and Mxc mutants tested showed elevated levels of cytochrome c-553. This phenotype was more pronounced in the MxbM and MxbD mutants than in the Mxc mutants (Fig. 5). Levels of this protein are high enough in Mxd mutants that a band corresponding to cytochrome c-553 is visible (running at about 23 kDa) in Coomassie-blue-stained extracts of Mxd mutants (Fig. 4). The MxdR and MxdS mutants contained wild-type levels of MxaF and cytochrome cL and did not show elevated levels of cytochrome c-553 (Figs 4 and 5).

DISCUSSION

The complementation analysis of 208 new Mox mutants in \textit{M. extorquens} AM1 has identified 84 mutants whose mutations map to the Mxb, Mxc or Mxd loci. The mutants at the Mxb locus comprise at least two complementation groups, MxbM and MxbD. The site of the gene defective in MxbM mutants was mapped to a specific BglII site in the insert of p1130D (Fig. 1). MxbM mutants show the same complementation pattern as the MxbM mutants reported in \textit{M. organophilum} XX (W.-H. Fan & M. E. Lidstrom, unpublished; Machlin \textit{et al.}, 1987). The MxbD complementation group has been previously characterized in \textit{M. extorquens} AM1 (Nunn & Lidstrom, 1986a). However, the MxbD mutants from this new collection fall into two classes based on their ability to recombine with pALS1, suggesting that the gene defective in these mutants lies across the BamHI site in the insert of p1130D (Fig. 1). This is consistent with a previous analysis in which a Tn5 insertion affecting the MxbD complementation group was mapped 0.22 kb to the right of this BamHI site (Lee \textit{et al.}, 1991). In \textit{M. organophilum} XX another complementation group, MxbN, has been reported (Formerly VI-B; Machlin \textit{et al.}, 1987). However, both \textit{M. organophilum} XX MxbN and MxbD mutants show the same complementation pattern in our hands as the \textit{M. extorquens} AM1 MxbD mutants. It is possible that this discrepancy is due to differences in expression of genes from \textit{M. extorquens} AM1 clones in the \textit{M. organophilum} XX clones.

The MxbM and MxbD loci lie immediately adjacent to the \textit{pqgDGCBA} genes (Fig. 1) reported by Morris \textit{et al.} (1994). Part of this \textit{pqg} region has been sequenced and a putative promoter has been identified (Morriss \textit{et al.}, 1994; Ramamoorthi & Lidstrom, 1995). However, the direction of transcription of genes at the Mxb locus was not determined in this study.

The 30 mutants in the Mxc region fell into three complementation groups: MxcB, MxcQ and MxcE. The MxcQ complementation group showed the same complementation pattern as MxcQ mutants in \textit{M. organophilum} XX (W.-H. Fan & M. E. Lidstrom, unpublished). The MxcE complementation group has already been described for \textit{M. extorquens} AM1 (Nunn & Lidstrom, 1986a). The presence of a third gene in the Mx locus of \textit{M. extorquens} AM1 has been proposed.
organophiltlm XX, called moxU, was implied by Xu et al. (1993), although the complementation or mapping of this gene has not been reported. A Tn5 insertion that affects the Mxc complementation groups has been reported in M. extorquens AM1 and was mapped 0-4 kb from the left-hand HindIII site of this region (Lee et al., 1991), which would place this insertion within the MxcB region. The complementation data reported here suggest that these genes are located at the left side of the insert in pDN202 (Fig. 2), and that the order, from left to right in Fig. 2, is MxcB, MxcQ, MxcE. The direction of transcription for the genes defective in MxcB and MxcQ mutants could be inferred by the orientation of the complementing subclones to be from left to right (Fig. 2). The direction of transcription for the gene defective in MxcE mutants was not determined. The proximity of MxcB, MxcQ and MxcE loci and the orientation of MxcB and MxcQ, suggest that the respective genes may be cotranscribed.

The class of mutants known as MxcQE are both MxcQ and MxcE. These strains may be double mutants, or they may contain polar mutations that affect cotranscribed genes. If the latter were the case, this would indicate that the genes defective in both MxcQ and MxcE are cotranscribed.

The complementation groups at the Mxb and Mxc loci appear to be required for proper expression of MxaF and cytochrome c₅₅₃ protein. There is evidence that these complementation groups are required at the level of transcription: Mxb and Mxc mutants do not show transcription of a reporter gene fused to the mxaF promoter region (Morris & Lidstrom, 1992b; Xu et al., 1993; A. L. Springer & M. E. Lidstrom, unpublished). In addition, all of the Mxb and Mxc mutant classes have elevated levels of the cytochrome c-553 protein. This phenotype is more pronounced in the MxbM and MxbD mutants than the Mxb, MxcQ or MxcE mutants (Fig. 5). Our data imply that the expression of this cytochrome is negatively regulated, either directly or indirectly, by the Mxb and Mxc gene products.

Mox mutants at a new locus, Mxd, are also reported here. The DNA restriction pattern of the clones complementing Mxd mutants does not overlap with that of any known Mox regions of M. extorquens AM1. Thus, the physical relationship of the Mxd to other Mox regions could not be determined. Complementation analysis showed that there are at least two complementation groups in this region: MxdR and MxdS. Phenotypic characterization of these mutants showed normal levels of MDH, cytochrome c₅₅₃ and cytochrome c-553 polypeptides. This indicates that the genes defective in MxdR and MxdS mutants are not required for production of these proteins. The functions of MxdR and MxdS are unknown, but they may be required for some form of post-translational processing of the MDH complex.

Complementation analyses of these and previously described mutants have so far identified 20 Mox complementation groups in M. extorquens AM1. However, there are several Mox genes, identified by sequencing, for which mutants were not isolated in this study: mxaJ, mxaR, mxaS, mxaD, pqqD and pqqB (Anderson et al., 1990; van Spanning et al., 1991; Morris et al., 1994). Mutants have been constructed in mxaJ and mxaR in P. denitrificans (van Spanning et al., 1991), and a Tn5 insertion has been isolated in mxaJ of M. extorquens AM1 (Lee et al., 1991). These mutants have been shown to be defective in MDH activity. Mutants have also been isolated in pqqB of M. organophilum DSM760 (Morris et al., 1994). Therefore, there are at least 26 complementation groups that have been shown to be required for methanol oxidation (Table 2). Although some complementation groups have not

### Table 2. Functional roles of methanol oxidation genes in M. extorquens AM1

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene (or complementation group)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Structure</td>
<td>mxaF (large subunit)</td>
<td>Nunn &amp; Lidstrom (1986b)</td>
</tr>
<tr>
<td></td>
<td>mxa1 (small subunit)</td>
<td>Anderson &amp; Lidstrom (1988)</td>
</tr>
<tr>
<td></td>
<td>mxaG (cytochrome c₅₅₃)</td>
<td>Nunn &amp; Lidstrom (1986b)</td>
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<td>Morris et al. (1994)</td>
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<td>pqqEF</td>
<td>Morris et al. (1994)</td>
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<td>MxaB</td>
<td>Morris &amp; Lidstrom (1992a)</td>
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<td></td>
<td>MxbM, MxbD</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>MxcBQE</td>
<td>This study</td>
</tr>
<tr>
<td>Unknown</td>
<td>mxaF</td>
<td>Anderson &amp; Lidstrom (1988)</td>
</tr>
<tr>
<td></td>
<td>mxaRS</td>
<td>van Spanning et al. (1991); C. J. Morris &amp; M. E. Lidstrom, unpublished</td>
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<tr>
<td></td>
<td>mxaD</td>
<td>C. J. Morris &amp; M. E. Lidstrom, unpublished</td>
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<td>MxdRS</td>
<td>This study</td>
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</table>
been identified from this mutagenesis, sequence and Tn5 analyses performed so far have indicated the presence of \textit{mxaF}, \textit{mxaR}, \textit{mxaD} and \textit{pqqD} in \textit{M. extorquens} AM1 (Anderson et al., 1990; Lee et al., 1991; Morris et al., 1994; C. J. Morris & M. E. Lidstrom, unpublished). It is possible that redundant functions in \textit{M. extorquens} AM1 prevent some genes from yielding a Mox phenotype; however it may simply be that a larger-scale mutagenesis is required to yield mutants in all 26 complementation groups.

The large number of genes required for the oxidation of methanol to formaldehyde underscores the complexity of this process. These genes include those required for synthesis of PQQ and for the insertion of Ca\textsuperscript{2+} into MDH. Genes or complementation groups whose function is known are shown in Table 2. Current data indicate that most of the groups of unknown function may be required for proper assembly of the MDH complex. In addition, there are at least six genes that function as positive regulators of transcription of \textit{mxaF} (Table 2). Thus, the regulation of methanol oxidation appears to be fairly complex, probably allowing MDH levels to respond to slight changes in the environment (Lidstrom, 1991; Xu et al., 1993). Further analysis of Mox regulatory mutants should yield insight into this response.

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