Characterization of transposon insertion mutants of *Methylobacterium extorquens* AM1 (*Methylobacterium* strain AM1) which are defective in methanol oxidation

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Three previously reported Tn5 mutants of *Methylobacterium extorquens* AM1, Cou-1, Cou-3 and Cou-6, which are able to grow on methylamine but not methanol, were characterized by biochemical analyses and complementation tests using two genomic libraries of *M. extorquens* AM1. We have designated the genes defective in these mutants as cou-1, cou-3 and cou-6 and mapped the site of Tn5 insertion in each. Biochemical results showed that two of these methanol oxidation (Mox) mutants, Cou-1 and Cou-3, are phenotypically similar to the previously identified MoxE class of mutant while Cou-6 resembled the MoxD class. Complementation tests and mapping the site of the Tn5 insertions indicated that cou-1 is another Mox gene linked to moxE and that cou-6 is linked to moxD. The Tn5 insertion in cou-3 mapped within the moxJ gene and therefore is the first detected mutation of the moxJ gene which was identified from expression studies. A MoxE mutant of *M. extorquens* AM1 was complemented by two different cosmid clones; one carried the moxE gene and the other contained two mox gene clusters, moxFJGI and moxAKLB. Hybridization experiments indicated that the moxE gene was not present on this latter clone, and it must therefore encode a gene capable of suppressing a MoxE mutation.

Introduction

*Methylobacterium extorquens* AM1 is a pink-pigmented facultative methylotroph which is able to grow on reduced C₁ compounds such as methanol and methylamine as well as on multicarbon compounds, including ethanol and succinate. Methanol and ethanol are oxidized by methanol dehydrogenase, a periplasmic quinoprotein which is composed of two alpha subunits (60 kDa) and two beta subunits (8.4 kDa) arranged in an alpha₂beta₂ configuration (Nunn et al., 1989). Two molecules of pyrroloquinoline quinone (PQQ) are bound non-covalently to each enzyme molecule. There is evidence that electrons are transferred from methanol dehydrogenase to cytochrome c₅₉, an unusual c-type cytochrome which is specifically involved in methanol oxidation (see Anthony, 1986). *M. extorquens* AM1 contains a second c-type cytochrome (c₅₉), which may be involved in electron transport from methanol and/or methylamine (Beardmore-Gray et al., 1983; Fukumori & Yamanaka, 1987).

Following investigations of the biochemistry and genetics of mutants of *M. extorquens* AM1 and *M. organophilum* which were unable to oxidize methanol, Lidstrom and coworkers identified and partially mapped at least 17 genes (rnox genes) which are involved in methanol oxidation (Nunn & Lidstrom, 1986a, b; Anderson & Lidstrom, 1988; Nunn et al., 1989; Lidstrom & Stirling, 1990). The moxF and moxl genes which encode the alpha and beta subunits of methanol dehydrogenase are closely linked to moxG, the structural gene for cytochrome c₅₉, and moxJ, which encodes a 30 kDa protein of unknown function. These genes are arranged in the order (5' to 3') moxFJGI. Three genes — moxA, K and L (formerly moxA1, moxA2 and moxA3) — which are closely linked to each other, are thought to be required for the association of PQQ with methanol dehydrogenase. These three genes are linked to moxB, a putative regulatory gene. Another six genes, moxC, P, O, M, N and D, are closely linked; three (moxC, P and O) are required for PQQ synthesis and the rest have unknown functions. Another gene, moxH, is unlinked to

*Abbreviation:* PQQ, pyrroloquinoline quinone.
this cluster, but is also necessary for PQQ synthesis. A final mox region contains a further two genes of unknown function, moxE and Q.

The mutants used by Nunn & Lidstrom (1986a, b) were isolated using UV and chemical mutagenesis. In this paper we describe the biochemical characterization of three Tn5 insertion mutants of *M. extorquens* AM1 which are defective in methanol oxidation. We have mapped the site of the Tn5 insertion in each one and complemented them using cosmid clones from two genomic libraries. Our results demonstrate that one of the mutants is defective in the *moxJ* gene. The second is probably mutated in *moxN* and the third may have a lesion in *moxQ*.

**Methods**

**Bacterial strains.** The bacterial strains and plasmids used in this study are shown in Table 1.

**Growth of organisms.** *M. extorquens* AM1 was grown on MacLennan's medium as described by Tatra & Goodwin (1983). *Escherichia coli* strains were grown in either LB medium (Maniatis et al., 1982) or minimal medium (Vogel & Bonner, 1956). Strains carrying plasmids and/or transposons were grown in the presence of the appropriate antibiotics.

Antibiotics in the growth medium were used at the following final concentrations: oxytetracycline hydrochloride (Sigma), 20 µg ml⁻¹ for *E. coli*, 10 µg ml⁻¹ for *M. extorquens* AM1; kanamycin sulphate (Sigma), 100 µg ml⁻¹ for *M. extorquens* AM1, 20 µg ml⁻¹ in LB medium and 50 µg ml⁻¹ in minimal medium for *E. coli*.

**Enzyme assays.** Bacteria were grown on media containing methanol and methylamine as carbon sources in order to induce methanol dehydrogenase to maximum levels. Cell extracts were prepared as described by Tatra & Goodwin (1985); methanol dehydrogenase was assayed by the method of Dunstan et al. (1972) as modified by Tatra & Goodwin (1985).

**Partial purification of cytochrome.** Crude extracts were acid treated, then passed down a DEAE-cellulose column to separate cytochromes *c*₁ and *c*₂ as described by O'Keeffe & Anthony (1980); the resulting fractions were scanned for the presence of reduced cytochrome *c* after the addition of sodium dithionite.

**SDS gel electrophoresis.** Whole cells (approx. 500 µg wet weight), grown on medium containing methanol and methylamine, were boiled for 5 min with sample buffer and then electrophoresed on SDS polyacrylamide gels (Laemmli, 1970).

**Western blotting.** Duplicate samples of lysed whole cells were electrophoresed on a 12% (w/v) SDS polyacrylamide gel. One set of duplicate samples was stained with PAGE-blue and the other set was blotted onto a Hybond nylon membrane as described by Nunn & Lidstrom (1986a). The blot was then incubated with antibody raised against purified methanol dehydrogenase prepared as described by Nunn & Lidstrom (1986b) (this was a gift from D. Nunn). Bound antibody was detected using the Bio-Rad Immunoblot GAR-HPR assay. Lysates of wild-type *M. extorquens* AM1 were used as a positive control; a lysate of *E. coli* was used as a negative control to check that in our system the methanol dehydrogenase antibody preparation did not cross-react with a 58 kDa protein (the Gram-negative 'common antigen') which is found in many Gram-negative bacteria and can be confused with methanol dehydrogenase (Nunn & Lidstrom, 1986b). We did not detect this protein in our experiments.

**Haem stain.** Samples of lysed whole cells were electrophoresed on a 15% SDS polyacrylamide gel, then cytochromes were detected using o-dianisidine, as described by Francis & Becker (1984). After photography of the haem stain the gel was stained with PAGE-blue.

**Complementation of *C₁*-negative mutants of *M. extorquens* AM1.** Two genomic libraries of *M. extorquens* AM1 were used. One was constructed by cloning fragments from a partial Sau3A digest of *M. extorquens* AM1 chromosomal DNA into the BglII site of cosmid vector pLA2917. The recombinant DNA was packaged into λ heads and then introduced into *E. coli* S17-1. Complementation tests were carried out as described by Lyon et al. (1988) on MacLennan's minimal medium supplemented with methanol and tetracycline.

The HindIII library constructed by Fulton et al. (1984) in cosmid vector pVK100 was also used. *E. coli* HB101 was the host for this library and triparental matings were performed as described by Stone & Goodwin (1989).

For complementation of MoxD and MoxE mutants, two different strains carrying each mutation were used. UV9 (MoxD) and AA31 (MoxE) were used with the Sau3A bank. Complementation by the HindIII library was carried out with UV27 (MoxD) and AA32 (MoxE).

**DNA manipulation and isolation.** *M. extorquens* AM1 chromosomal DNA was prepared by the methods of Scott et al. (1981) and Fulton et al. (1984). Large-scale plasmid DNA extractions from *E. coli* were done as described by Nayudu & Holloway (1981). Rapid plasmid extractions were carried out using the boiling method described in Maniatis et al. (1982). Transformation of plasmid DNA was performed according to Maniatis et al. (1982) using competent cells prepared by the method of Cohen et al. (1972). Restriction enzymes were obtained from Promega Biotech and used according to the manufacturer's instructions.

Agarose gel electrophoresis was performed using 0.7% agarose in Tris/acetate buffer (Maniatis et al., 1982). DNA fragments used for hybridization and subcloning were purified from low-melting-temperature agarose gels as in Maniatis et al. (1982).

**DNA–DNA hybridizations.** DNA was transferred from agarose gels to Hybond-N nylon membranes (Amersham) by the method of Smith & Summers (1980). DNA preparations used as probes were nick-translated according to the method of Rigby et al. (1977). The blots were treated in 50% (v/v) formamide, 4 × SSPE (Maniatis et al., 1982), 0.1% (w/v) SDS, 0.5% (v/v) Blotto (10%, w/v, skim milk powder plus 0.2%, w/v, sodium azide) and 50 µg ml⁻¹ of denatured herring sperm DNA for 2 h at 37°C prior to the addition of nick-translated probe. Hybridization was carried out at 37°C for 24 h. The blots were then washed twice in 2 × SSC (Maniatis et al., 1982), 0.1% SDS at 65°C for a total of 40 min. Another wash of higher stringency was performed in 0.1 × SSC, 0.1% SDS at 65°C for 20 min. Autoradiography of the blots was done at -70°C for 12-24 h.

**Tn5 mutagenesis of pMO1917.** The plasmid was transferred from *E. coli* S17-1 into *E. coli* C21, which harbours Tn5, by delayed patch mating on LB medium for 4 h at 37°C then streaking onto kanamycin and tetracycline selective medium and incubating at 37°C for 24 h. Several of these Km⁸ Tc⁸ exconjugants were purified and mated with *E. coli* DH1 in order to select for pMO1917 with Tn5 inserted. Overnight cultures of *E. coli* C21(pMO1917) and *E. coli* DH1 were mixed in a ratio of 2:5:1 and passed through a membrane filter (Gelman; 0.45 µm pore size and 25 mm diameter). The filter was incubated on LB medium at 37°C for 4 h. Cells were washed off with 1 ml of saline and 0.2 ml aliquots were plated onto minimal medium supplemented with 0.5 mm-thiamin and kanamycin then incubated for 4 d at 37°C. Plasmid DNA was prepared from these Km⁸ exconju-
Mox mutants of Methylobacterium extorquens AM1

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties*</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylobacterium extorquens AM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>Peel &amp; Quayle (1961)</td>
</tr>
<tr>
<td>Cou-1</td>
<td>cou-1::Tn5</td>
<td>Whitta et al. (1985)</td>
</tr>
<tr>
<td>Cou-3</td>
<td>cou-3::Tn5</td>
<td>Whitta et al. (1985)</td>
</tr>
<tr>
<td>Cou-6</td>
<td>cou-6::Tn5</td>
<td>Whitta et al. (1985)</td>
</tr>
<tr>
<td>UV27, UV9</td>
<td>moxG</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
</tr>
<tr>
<td>AA32, AA31</td>
<td>moxE</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
</tr>
<tr>
<td>UV10</td>
<td>moxG</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
</tr>
<tr>
<td>Escherichia coli</td>
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<td></td>
</tr>
<tr>
<td>HB101</td>
<td>F' hsdR17 (rpsL20 yaiS5 mit5 Tn5 supE44 thi-1 leuB6 k')</td>
<td>Fulton et al. (1984)</td>
</tr>
<tr>
<td>S17-1</td>
<td>pro hsdR17 hsdM' thi-1 recA4 ara-14 proAB lacY1 galK2 rpsL20 yaiS5 mit5 Tn5 supE44 thi-1 leuB6 k'</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>CZ1</td>
<td>his::Tn5 derivative of S17-1</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>DH1</td>
<td>recA1 endA1 gyrA96 thi-1 supE44 relA2 F- hsdR17 (rk m:) supE44 relA2 F-</td>
<td>Holloway Collection</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVK100</td>
<td>Te' Km' cos</td>
<td>Knauf &amp; Nester (1982)</td>
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<tr>
<td>pRK2013</td>
<td>Km' ColE1 replicon</td>
<td>Figurski &amp; Helinski (1979)</td>
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<td>pLA2917</td>
<td>Km' Te' cos</td>
<td>Allen &amp; Hanson (1985)</td>
</tr>
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<td>pRK310</td>
<td>Te' lacPOZ</td>
<td>Ditta et al. (1985)</td>
</tr>
<tr>
<td>pMO120836</td>
<td>Ccou-3 CmoxF CmoxG CmoxA CmoxK CmoxL CmoxB Ccoul-3 transposon with CmoxE CmoxF cos</td>
<td>This paper</td>
</tr>
<tr>
<td>pMO121511</td>
<td>CmoxC CmoxD Ccou-6 Te' cos</td>
<td>This paper</td>
</tr>
<tr>
<td>pMO120004</td>
<td>Ccoul-3 transposon with CmoxE CmoxF Ccoul-3 CmoxA CmoxK CmoxL CmoxB Ccoul-3 transposon with CmoxE CmoxF cos</td>
<td>This paper</td>
</tr>
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<td>pMO1917</td>
<td>Ccoul-3 CmoxD Te' A, 6-65 kb HindIII-PstI fragment of pMO121511</td>
<td>This paper</td>
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<tr>
<td>pMO1918</td>
<td>CmoxD Te' A, 4-0 kb HindIII-BamHI fragment of pMO121511</td>
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<tr>
<td>pMO1919</td>
<td>CmoxD Te' A, 4-0 kb HindIII-BamHI fragment of pMO121511</td>
<td>This paper</td>
</tr>
<tr>
<td>pHIN-E</td>
<td>CmoxE Ccoul-1 Te' A, 4-0 kb HindIII-BamHI fragment of pMO121511 sub-cloned into pRK310, Te' A</td>
<td>This paper</td>
</tr>
<tr>
<td>pHIN-FG</td>
<td>CmoxF Ccoul-3 Te' A</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
</tr>
<tr>
<td>pHIN-D</td>
<td>CmoxD Ccoul-6 Te' A</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
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<td>pORF9</td>
<td>0-85 kb EcoRI-BamHI fragment of moxF gene</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
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<td>pYK310</td>
<td>0-90 kb PstI-BamHI fragment of moxA gene</td>
<td>Kim &amp; Lidstrom (1989)</td>
</tr>
<tr>
<td>pYK340</td>
<td>1-86 kb SstI fragment of moxG gene</td>
<td>M. Lidstrom (unpublished)</td>
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</table>

*To denote complementation, we have used the nomenclature described in Moore et al. (1983) by putting the prefix C (for complementation) followed by the gene symbol of the M. extorquens AM1 mutant that was complemented by the particular clone.

Gants by the boiling method and then used to transform competent E. coli S17-1 by selecting for kanamycin resistance. Exconjugants were picked and grown in microtitre trays and screened for their abilities to complement UV9 and Cou-6.

Results

Biochemical analysis of Tn5 insertion mutants

The mutants used in this study were isolated by Whitta et al. (1985) and designated Cou-1, Cou-3 and Cou-6. They are able to grow on methylamine and succinate but not on methanol or ethanol, indicating that they are all defective in methanol oxidation. Growth was not observed on methanol medium supplemented with the methanol dehydrogenase cofactor PQQ. Extracts of these mutants had no detectable methanol dehydrogenase activity. Furthermore, the alpha subunit of methanol dehydrogenase was not detected in cell lysates of any of the mutants when SDS polyacrylamide gel electrophoresis and Western blotting was carried out, the latter using an antibody raised against purified methanol dehydrogenase. When lysates of wild-type bacteria were used one band was observed, namely the 60 kDa alpha subunit of methanol dehydrogenase.

Crude extracts of all three mutants contained cytochromes which eluted from DEAE-cellulose columns in the positions expected of cytochromes cH and cL. SDS polyacrylamide gel electrophoresis of whole-cell lysates followed by staining for haem proteins confirmed the presence of cytochrome cH but cytochrome cL (molecular mass on SDS-polyacrylamide gels approximately 20-9 kDa) was not observed in extracts of the mutants (Fig. 1). Instead a haem protein of about 23 kDa was present in
small amounts in Cou-1 and Cou-3 and in larger amounts in Cou-6. We also observed this 23 kDa cytochrome in large amounts in UV27, a MoxD mutant, and in trace amounts in AA32, a MoxE mutant, confirming the results of Nunn & Lidstrom (1986b). Day et al. (1990) have recently shown that the 23 kDa cytochrome, designated cytochrome c-553, is a novel c-type cytochrome which has no structural relationship to cytochrome cL. Its function is unknown. Thus phenotypically Cou-1 and Cou-3 resemble the MoxE class of mutants and Cou-6 the MoxD class. These results are summarized in Table 2.

Tn5 mapping and complementation tests
Individual clones from HindIII and Sau3A gene libraries of M. extorquens AM1 DNA known to complement previously characterized Mox mutants were tested for their ability to complement Cou-1, Cou-3 and Cou-6, with previously characterized MoxD and E mutants being included as controls (Table 3).

(i) Cou-1. Cou-1 and a MoxE mutant, AA32, were found to be biochemically similar according to the tests performed on them. They were both complemented by the same clone from the HindIII library, namely pHIN-E. This plasmid carries a 7.6 kb HindIII fragment known as HINDIII-E. Another MoxE mutant, AA31, was complemented by two clones from the Sau3A library, pMO122004 and pMO120836. The latter also complemented mutants of the MoxA, K, L, B, F and G classes. However, Cou-1 was not complemented by either pMO122004 or pMO120836, suggesting that Cou-1 does not have a defect in the moxE gene.

As these results indicate that the defects in Cou-1 and moxE are linked on HINDIII-E, this fragment was isolated from pHIN-E and used to probe chromosomal
Table 3. Complementation of Cou and Mox mutants

<table>
<thead>
<tr>
<th>Cosmid clones</th>
<th>Mutants:</th>
<th>MoxE class</th>
<th>MoxD class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cou-1</td>
<td>Cou-3</td>
<td>AA31</td>
</tr>
<tr>
<td>pMO120836*</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pHIN-FG</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>pMO122004</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pHIN-E</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>pMO121511†</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>pMO1917</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pMO1919</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>pHIN-D</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not done.
* This clone is also able to complement mutants defective in moxA, K, L, B, F and G.
† This clone is also capable of complementing a mutation in moxC.

digests of Cou-1, wild-type *M. extorquens* AM1 and digests of the cosmids pMO120836 and pMO122004. As shown in Fig. 2, the HINDIII-E fragment hybridized to a 7-6 kb HindIII fragment of the wild type DNA (lane 2) and to two HindIII fragments of Cou-1 (lane 5), confirming that the Tn5 insertion in Cou-1 was within HINDIII-E. Consideration of the results of probing BamHI and BamHI/HindIII digests of Cou-1 and wild-type DNA with HINDIII-E indicated that the Tn5 insertion in Cou-1 mapped within 0-4 kb of the HindIII site of HINDIII-E (Fig. 3c). In contrast, Nunn & Lidstrom (1986a) mapped the moxE gene to about 2 kb from the same site. Furthermore the HINDIII-E probe hybridized to a 7-5 kb HindIII fragment of pMO122004 (Fig. 2, lane 11, fragment arrowed). Approximately 0-37 kb of this 7-5 kb fragment was vector DNA and thus only about 7-13 kb of HINDIII-E was present in pMO122004; the remaining 0-47 kb of HINDIII-E must therefore contain at least part of the gene which complements Cou-1.

Even though a MoxE mutant, AA31, was complemented by pMO120836, we could not demonstrate any homology between the chromosomal insert of pMO120836 and HINDIII-E (Fig. 2, lanes 8–10). Hybridization was observed between the HINDIII-E probe and the largest fragment of pMO120836, which is mainly vector DNA. However, since this probe also has homology with the large fragment of pMO122004 (lanes 11–13), the hybridization can be attributed to contamination of the HINDIII-E fragment by DNA from the vector, pVK100, which, like pLA2917, is derived from plasmid RK2.

When labelled pMO120836 was used to probe a HindIII digest of wild-type *M. extorquens* AM1 DNA, homology was shown with three chromosomal fragments, none of which corresponded to HINDIII-E (Fig. 4, lane 2). The largest fragment to which pMO120836 hybridized was HINDIII-AB (which carries the moxA, K, L and B genes) and the smallest was HINDIII-FG (which carries the moxF, J, G and I genes). The other chromosomal fragment was about 11-6 kb, whereas HINDIII-E is only 7-6 kb. In addition we have shown that pMO120836 carried the HINDIII-FG fragment and part of the HINDIII-AB fragment (Fig. 3a; H1 to H2 and H2 to H3 respectively) by probing with clones carrying moxF (pORF9), moxA (pYK310) and moxL (pYK340) (data not shown). These three clones were kindly provided by Dr M. Lidstrom. Therefore complementation of AA31 by pMO120836 could not be due to the presence of the moxE gene on this cosmids and it must contain a gene which is capable of suppressing the MoxE mutation.

(ii) Cou-3. Although its biochemical characteristics were similar to the MoxE group of mutants, Cou-3 was complemented by pHIN-FG from the HindIII gene library and by pMO120836 from the Sau3A gene library. As shown in Fig. 3(a), pMO120836 consists of the HINDIII-FG region, which is flanked by part of HINDIII-AB on its right and part of an unknown 11-6 kb HindIII fragment on its left. Comparison of the HindIII digests of wild-type *M. extorquens* AM1 and Cou-3 after probing with pMO120836 (Fig. 4, lanes 2 and 5), showed that in Cou-3 Tn5 is present on the HINDIII-FG fragment. The insert was located about 1-3 kb upstream from the BamHI site of HINDIII-FG as indicated in Fig. 3(a). Hence, the insertion is in the middle of the 1-0 kb XhoI2-XhoI1 fragment. Anderson & Lidstrom (1988) have shown that the region from the XhoI1 site to the BamHI site of HINDIII-FG codes for
four polypeptides which are the products of the $\textit{mox}F$, $J$, $G$ and $I$ genes. Part of the $\textit{mox}J$ gene, which encodes a 30 kDa protein of unknown function, extends into the 1.0 kb $XhoI_2$–$XhoI_3$ fragment. DNA sequence analysis by Nunn & Anthony (1989) and Anderson et al. (1990) has shown that the end of $\textit{mox}J$ is 865 bases from the $XhoI_2$ site, i.e. about 329 bases after the Tn5 insertion in cou-3. Thus, the Tn5 insertion in cou-3, which resembles MoxE-type mutants biochemically, is the first reported mutation of the $\textit{mox}J$ gene.

(iii) Cou-6. The mutant Cou-6 resembled the MoxD group of mutants phenotypically and both mutants were complemented by the same clones from the Sau3A library (pMO121511: see Table 3 and Fig. 3d) and the HindIII library (pHIN-D). Nunn & Lidstrom (1986a) generated a Tn5 marker exchange mutation which indicated that $\textit{mox}D$ mapped 1.6 kb to the left of the $\textit{BamHI}_1$ site of HINDIII-D. To map the Tn5 insertion of Cou-6, digests of wild-type $M. \textit{extorquens}$ AM1 and Cou-6 DNA were probed with pMO121511. The results are shown in Fig. 5. The Tn5 insertion was not on the 4-0 kb HindIII–$\textit{BamHI}$ fragment to which $\textit{mox}D$ mapped but was on the 2.65 kb $\textit{BamHI}$–PstI fragment which lies next to it (see Fig. 3d). The insertion is 0.22 kb to the right of this $\textit{BamHI}$ site. The HindIII–PstI, HindIII–$\textit{BamHI}$ and $\textit{BamHI}$–PstI fragments of the 6.65 kb HindIII–PstI fragment were subcloned into pRK310. The abilities of those subcloned fragments to complement UV9 and Cou-6 are summarized in Table 3. The 6.65 kb HindIII–PstI fragment (pMO1917) complemented both UV9 and Cou-6. However, the 4-0 kb HindIII–$\textit{BamHI}$ fragment...
Fig. 3. Restriction enzyme maps of cosmid clones: (a) pMO120836, (b) pMO122004, (c) pHIN-E and (d) pMO121511. Chromosomal Tn5 insertions, i.e. the insertions in the Cou mutants, are indicated by arrows. Tn5 insertions into the HindIII–PstI subclone of pMO12511, pMO1917, which prevent complementation are represented by open triangles. Insertions which allow complementation are indicated by solid triangles. The hatched region represents vector DNA. B, BamHI; H, HindIII; P, PstI; X, XhoI. On pMO120836, H1–H2 represents the HINDIII-FG region, and on pMO121511 H1–H2 represents the HINDIII-D region.

(pMO1918) complemented UV9 but not Cou-6, suggesting that the mutants are different. The gene defective in Cou-6 has a BamHI site in it, as shown by the absence of complementation of Cou-6 by the 2.65 kb BamHI-PstI fragment (pMO1919), although this result could also be explained if an upstream region necessary for expression of cou-6 is missing from this fragment. Tn5 insertions in the 6-65 kb HindIII–PstI fragment are shown in Fig. 3(d). Insertions covering 2-8 kb of DNA were unable to complement either UV9 or Cou-6. Over 1000 pMO1917::Tn5 isolates were screened; none was observed to complement one mutant but not the other. A complicating factor in complementation tests involving Cou-6 may have been recombination between Tn5 elements on the pMO1917::Tn5 isolates and the Tn5 inserted into the chromosome of the Cou-6 region. However, positive complementation was observed for both the Tn5 mutant Cou-6 and the UV-induced mutant UV9. Thus it can be concluded that cou-6 extends at least as far as the BamHI–PstI fragment (see Fig. 3(d)).

Fig. 4. Hybridization of pMO120836 probe to restriction enzyme digests of wild-type M. extorquens AM1 genomic DNA and Cou-3 genomic DNA. Lane 1 contains λ HindIII fragments used as size standards. The other lanes are (i) HindIII-digested wild-type M. extorquens AM1 (lane 2) and Cou-3 (lane 5); (ii) HindIII- and BamHI-digested wild-type M. extorquens AM1 (lane 3) and Cou-3 (lane 6); (iii) BamHI-digested wild-type M. extorquens AM1 (lane 4) and Cou-3 (lane 7).
Fig. 5. Hybridization of pMO121511 probe to restriction enzyme digests of wild-type *M. extorquens* AM1 and Cou-6 genomic DNAs. Lane 1 contains λ HindIII fragments used as size standards. Wild-type *M. extorquens* AM1 was digested with HindIII (lane 2), HindIII and BamHI (lane 3), BamHI (lane 4), BamHI and PstI (lane 5) and PstI (lane 6). Cou-6 was digested with HindIII (lane 7), HindIII and BamHI (lane 8), BamHI (lane 9), BamHI and PstI (lane 10) and PstI (lane 11).

**Discussion**

Our study of the three Mox mutants of *M. extorquens* AM1 has shown that the mutations which they represent have not been described for *M. extorquens* AM1. The position of the mutation in *cou-3* suggests that it is a MoxJ mutant. The function of the 30 kDa protein encoded by this gene is unknown, but since *Cou-3* lacks the methanol dehydrogenase alpha subunit and cytochrome cL, it is possible that the *moxJ* gene product may regulate synthesis of both these proteins. However, the inability to synthesize these proteins could also be due to a polar effect of the Tn5 insertion in *Cou-3* on expression of *moxF* and *moxG*. Indeed, it has previously been reported that Tn5 insertions in the *maxFG* region have polar effects both up- and downstream (Nunn & Lidstrom, 1986a).

Mox mutants of the related pink-pigmented facultative methylotroph, *Methylobacterium organophilum* XX, have been isolated by Machlin et al. (1988). Complementation of some of these mutants with cloned *M. extorquens* AM1 DNA led to the identification of some *mox* genes of which there are no equivalent mutants in *M. extorquens* AM1 (Lidstrom & Stirling, 1990). One such gene is *moxQ*. This gene was found to be linked to the *moxE* equivalent of *M. organophilum* XX. *Cou-1* could be the *moxQ* equivalent in *M. extorquens* AM1 even though MoxQ mutants synthesize the alpha subunit of methanol dehydrogenase and cytochrome cL (Bastien et
al., 1989), whereas Cou-1 does not. This difference may be due to a polar effect of the Tn5 insertion in Cou-1 on expression of moxE.

The Tn5 insertion in the last of these mutants, Cou-6, mapped close to the proposed site for the moxD gene. Complementation tests using various subclones suggest that cou-6 and moxD are separate genes. Transposon mutagenesis of the region shows that the two genes are probably co-transcribed since no Tn5 insertions were detected that will complement one but not the other mutant. cou-6 maps further away from moxC, which is on the HindIII fragment to the left of HindIII-D. Another gene, moxN, is linked to moxD mutants in M. organophilum XX and there is evidence that these two genes are cotranscribed (Machlin et al., 1988; Bastien et al., 1989).

Mutants of M. extorquens AM1 which are defective in moxF have not been described but Lidstrom & Stirling (1990) proposed that the M. extorquens AM1 moxD gene is linked to a moxN gene; the order of these genes is unknown. cou-6 may be the moxN gene of M. extorquens AM1 although Cou-6 does not share the same phenotype as the M. organophilum XX MoxN mutant, which contains cytochrome c1 (Machlin et al., 1988). These differences may be due to a polar effect of the Tn5 insertion in Cou-6. Until the relationship of the cou genes to the mox genes can be firmly established it will be best to retain the separate gene nomenclature.

Our complementation tests have been done with two genomic libraries. This proved to be advantageous as it was observed that certain mutants could be complemented by clones from one library but not the other. This was also found to be the case with M. organophilum XX (Machlin et al., 1988). The Sau3A genomic library was helpful in determining linkage of some of the Mox genes. However, in our experience caution is necessary when interpreting the data from complementation tests. The complementation results using pMO120836 suggested that moxF, J, G, I was linked to moxA, K, L, B and moxE. Linkage of moxF, G, J, I to moxA, K, L, B was verified by hybridization of pMO120836 to M. extorquens AM1 moxF, moxA and moxL DNA, which yielded bands that correspond to HindIII-FG and HindIII-AB. However, restriction enzyme analysis and Southern hybridization showed that the moxE gene was not linked to the other genes. Hybridization results using pMO122004 and pMO120836 as probes against SpeI-digested genomic DNA of M. extorquens AM1 separated by pulsed-field gel electrophoresis showed that they were on different SpeI fragments (data not shown). This MoxE suppressor cannot be on either HindIII-FG or HindIII-AB as neither fragment complemented MoxE (Nunn & Lidstrom, 1986a). It is probable that this suppressor is located on the 11.6 kb HindIII fragment beside HindIII-FG.

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References


