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 *c*_L, an unusual *c*-type cytochrome which is specifically involved in methanol oxidation (see Anthony, 1986). *M. extorquens* AM1 contains a second *c*-type cytochrome (*c*_H), 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 (*mox* genes) which are involved in methanol oxidation (Nunn & Lidstrom, 1986*a, b*; Anderson & Lidstrom, 1988; Nunn *et al.*, 1989; Lidstrom & Stirling, 1990). The *moxF* and *moxI* genes which encode the alpha and beta subunits of methanol dehydrogenase are closely linked to *moxG*, the structural gene for cytochrome *c*_L, 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

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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 *c* cytochromes. Crude extracts were acid treated, then passed down a DEAE-cellulose column to separate cytochromes *c_H* and *c_L* 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 (1986a) (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 *Sau*3A digest of *M. extorquens* AM1 chromosomal DNA into the *Bgl*II 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 *Hind*III 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 *Sau*3A bank. Complementation by the *Hind*III 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 Biotec 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* CZ1, 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 Km^R Tc^R exconjugants were purified and mated with *E. coli* DH1 in order to select for pMO1917 with Tn5 inserted. Overnight cultures of *E. coli* CZ1(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 Km^R exconju-

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant properties*	Reference/source
<i>Methylobacterium extorquens</i> AM1		
Wild-type		Peel & Quayle (1961)
Cou-1	<i>cou-1::Tn5</i>	Whitta <i>et al.</i> (1985)
Cou-3	<i>cou-3::Tn5</i>	Whitta <i>et al.</i> (1985)
Cou-6	<i>cou-6::Tn5</i>	Whitta <i>et al.</i> (1985)
UV27, UV9	<i>moxD</i>	Nunn & Lidstrom (1986a)
AA32, AA31	<i>moxE</i>	Nunn & Lidstrom (1986a)
UV10	<i>moxG</i>	Nunn & Lidstrom (1986a)
<i>Escherichia coli</i>		
HB101	F ⁻ <i>hsdS20 (hsdR hsdM) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl supE44 thi-1 leuB6 λ⁻</i>	Fulton <i>et al.</i> (1984)
S17-1	<i>pro hsdR hsdM⁺ thi recA</i> (RP4-2, Tc ^r ::Mu, Km ^r ::Tn7, Tn1)	Boyer & Roulland-Dussoix (1969)
CZ1	<i>his::Tn5</i> derivative of S17-1	Simon <i>et al.</i> (1983)
DH1	<i>recA1 endA1 gyrA96 thi hsdR17 (r_k⁻ m_k⁺) supE44 relA λ⁻ F⁻</i>	Holloway Collection
Plasmids		
pVK100	Tc ^r Km ^r <i>cos</i>	Hanahan (1983)
pRK2013	Km ^r ColE1 replicon	Knauf & Nester (1982)
pLA2917	Km ^r Tc ^r <i>cos</i>	Figurski & Helinski (1979)
pRK310	Tc ^r <i>lacPOZ</i>	Allen & Hanson (1985)
pMO120836	<i>Ccou-3 CmoxF CmoxD CmoxA CmoxK CmoxL CmoxB CmoxE Tc^R cos</i>	Ditta <i>et al.</i> (1985)
pMO121511	<i>CmoxC CmoxD Ccou-6 Tc^R cos</i>	This paper
pMO122004	<i>CmoxE Tc^R cos</i>	This paper
pMO1917	<i>Ccou-6 CmoxD Tc^R, 6-65 kb HindIII-PstI fragment of pMO121511</i>	This paper
pMO1918	<i>CmoxD Tc^R, 4-0 kb HindIII-BamHI fragment of pMO121511</i>	This paper
pMO1919	<i>2-65 kb BamHI-PstI fragment of pMO121511 sub-cloned into pRK310, Tc^R</i>	This paper
pHIN-E	<i>CmoxE Ccou-1 Tc^R cos</i>	Nunn & Lidstrom (1986a)
pHIN-FG	<i>CmoxF CmoxD Ccou-3 Tc^R cos</i>	Nunn & Lidstrom (1986a)
pHIN-D	<i>CmoxD Ccou-6 Tc^R cos</i>	Nunn & Lidstrom (1986a)
pORF9	0.85 kb <i>EcoRI-BamHI</i> fragment of <i>moxF</i> gene	Nunn & Lidstrom (1986a)
pYK310	0.90 kb <i>PstI-BamHI</i> fragment of <i>moxA</i> gene	Kim & Lidstrom (1989)
pYK340	1.86 kb <i>SalI</i> fragment of <i>moxL</i> gene	M. Lidstrom (unpublished)

* 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 dehydrogen-

ase 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 *c_H* and *c_L*. SDS polyacrylamide gel electrophoresis of whole-cell lysates followed by staining for haem proteins confirmed the presence of cytochrome *c_H* but cytochrome *c_L* (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

Fig. 1. SDS polyacrylamide gels showing haem staining proteins in whole-cell lysates of wild-type and mutant strains of *M. extorquens* AM1. Lanes: 1, wild-type; 2, Cou-3; 3, UV27; 4, Cou-6; 5, UV10 (a MoxG mutant); 6, Cou-3; 7, wild-type; 8, AA32; 9, Cou-1.

Table 2. Summary of biochemical phenotypes of mutants

Mutant	MDH activity	MDH alpha subunit	Cyt. c_L	Cyt. c_{553}	Known Mox mutant of similar phenotype
Cou-1	—	—	—	+	MoxE
Cou-3	—	—	—	+	MoxE
Cou-6	—	—	—	+++	MoxD

MDH, methanol dehydrogenase.

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 c_L . 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 *Hind*III and *Sau*3A 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 *Hind*III library, namely pHIN-E. This plasmid carries a 7.6 kb *Hind*III fragment known as HINDIII-E. Another MoxE mutant, AA31, was complemented by two clones from the *Sau*3A 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

Cosmid clones	Mutants:						
	MoxE class					MoxD class	
	Cou-1	Cou-3	AA31	AA32	Cou-6	UV9	UV27
pMO120836*	—	+	+	ND	—	—	ND
pHIN-FG	—	+	ND	—	—	ND	—
pMO122004	—	—	+	ND	—	—	ND
pHIN-E	+	—	ND	+	—	ND	—
pMO121511†	—	—	—	ND	+	+	ND
pMO1917	ND	ND	ND	ND	+	+	ND
pMO1918	ND	ND	ND	ND	—	+	ND
pMO1919	ND	ND	ND	ND	—	—	ND
pHIN-D	—	—	ND	—	+	ND	+

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 *Hind*III fragment of the wild type DNA (lane 2) and to two *Hind*III fragments of Cou-1 (lane 5), confirming that the Tn5 insertion in Cou-1 was within HINDIII-E. Consideration of the results of probing *Bam*HI and *Bam*HI/*Hind*III 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 *Hind*III₁ 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 *Hind*III 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 *Hind*III digest of wild-type *M. extorquens* AM1 DNA, homology was shown with three chromosomal frag-

ments, 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 cosmid 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 *Hind*III gene library and by pMO120836 from the *Sau*3A 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 *Hind*III fragment on its left. Comparison of the *Hind*III 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 *Bam*HI₂ site of HINDIII-FG as indicated in Fig. 3(a). Hence, the insertion is in the middle of the 1.0 kb *Xho*I₂–*Xho*I₃ fragment. Anderson & Lidstrom (1988) have shown that the region from the *Xho*I₁ site to the *Bam*HI₂ site of HINDIII-FG codes for

Fig. 2. Hybridization of the HINDIII-E probe to restriction enzyme digests of wild-type *M. extorquens* AM1 genomic DNA, Cou-1 genomic DNA, pMO120836 and pMO122004. Lanes 1 and 14 contain λ HindIII fragments used as size standards. The other lanes are (i) HindIII-digested wild-type *M. extorquens* AM1 (lane 2), Cou-1 (lane 5), pMO120836 (lane 8) and pMO122004 (lane 11); (ii) HindIII- and BamHI-digested wild-type *M. extorquens* AM1 (lane 3), Cou-1 (lane 6), pMO120836 (lane 9) and pMO122004 (lane 12); (iii) BamHI-digested wild-type *M. extorquens* AM1 (lane 4), Cou-1 (lane 7), pMO120836 (lane 10) and pMO122004 (lane 13).

four polypeptides which are the products of the *moxF*, *J*, *G* and *I* genes. Part of the *moxJ* 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 *moxJ* 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 *moxJ* 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. 3*d*) and the *HindIII* library (pHIN-D). Nunn & Lidstrom (1986*a*)

generated a Tn5 marker exchange mutation which indicated that *moxD* mapped 1.6 kb to the left of the *Bam*HI₁ site of HINDIII-D. To map the Tn5 insertion of Cou-6, digests of wild-type *M. 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*–*Bam*HI fragment to which *moxD* mapped but was on the 2.65 kb *Bam*HI–*Pst*I fragment which lies next to it (see Fig. 3*d*). The insertion is 0.22 kb to the right of this *Bam*HI site. The *HindIII*–*Pst*I, *HindIII*–*Bam*HI and *Bam*HI–*Pst*I fragments of the 6.65 kb *HindIII*–*Pst*I 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*–*Pst*I fragment (pMO1917) complemented both UV9 and Cou-6. However, the 4.0 kb *HindIII*–*Bam*I 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 *Hind*III-*Pst*I 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, *Bam*HI; H, *Hind*III; P, *Pst*I; X, *Xho*I. On pMO120836, H₁-H₂ represents the *HIND*III-FG region, and on pMO121511 H₁-H₂ represents the *HIND*III-D region.

(pMO1918) complemented UV9 but not Cou-6, suggesting that the mutants are different. The gene defective in Cou-6 has a *Bam*HI site in it, as shown by the absence of complementation of Cou-6 by the 2.65 kb *Bam*HI-*Pst*I 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 *Hind*III-*Pst*I 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 *Bam*HI-*Pst*I fragment (see Fig. 3d).

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 *Hind*III fragments used as size standards. The other lanes are (i) *Hind*III-digested wild-type *M. extorquens* AM1 (lane 2) and Cou-3 (lane 5); (ii) *Hind*III- and *Bam*HI-digested wild-type *M. extorquens* AM1 (lane 3) and Cou-3 (lane 6); (iii) *Bam*HI-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 c_L , 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 c_L (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 *moxN* 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 *c_L* (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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