Localization of a Pyrroloquinoline Quinone Biosynthesis Gene Near the Methanol Dehydrogenase Structural Gene in *Methylobacterium organophilum* DSM 760

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A partial *Sau*3AI genomic bank of *Methylobacterium organophilum* DSM 760 was constructed in the cosmid pSUP106 and *moxF*, the structural gene for methanol dehydrogenase, was isolated. In *M. organophilum*, pSUP106 behaves as a suicide plasmid. This property was used to insert Tn5 into the bacterial chromosome, in the vicinity of *moxF*, by marker exchange. Mobilization of the Tn5-labelled chromosomal region by a broad-host-range plasmid, pJB3J1 (an R68-45 derivative), allowed the selection of several large R' hybrid plasmids in *Escherichia coli* HB101. Most of them were able to complement both mutants of the *moxF* region and mutant MTM1, the first mutant of the pyrroloquinoline quinone (PQQ) biosynthesis pathway in *M. organophilum*. The gene involved, *pqqA*, was subcloned and localized.

INTRODUCTION

Methanol dehydrogenase (MDH), which catalyses the oxidation of methanol to formaldehyde, is a key enzyme in methylotrophic bacteria utilizing methanol, either as a primary substrate or after methane oxidation. Methanol dehydrogenase is a quinoprotein, an enzyme with pyrroloquinoline quinone (PQQ) as the prosthetic group. Other quinoproteins include bacterial enzymes such as glucose dehydrogenase from *Acinetobacter calcoaceticus* (Duine *et al.*, 1979), alcohol dehydrogenase from *Pseudomonas aeruginosa* (Groen *et al.*, 1984) and methylamine dehydrogenase from methylotrophic bacteria (Van der Meer *et al.*, 1987). PQQ-dependent enzymes have also been found in mammals, e.g. the amine oxidase from bovine serum (Lobenstein-Verbeek *et al.*, 1984) or the lysyl oxidase from human placenta (Van der Meer & Duine, 1986). Information about PQQ and quinoproteins has recently been reviewed (Duine *et al.*, 1986).

The PQQ-dependent MDH of methylotrophs transfers electrons at the level of cytochrome *c* (Anthony, 1982). In *Methylobacterium* sp. AM1, an organism closely related to *Methylobacterium organophilum*, two cytochromes *c* have been described (O'Keefe & Anthony, 1980): cytochrome *c_L*, with a low isoelectric point (pI 4.2) and a high molecular mass (20.9 kDa), and cytochrome *c_H*, with a high pI (8.8) and a low molecular mass (11 kDa). In *M. organophilum* DSM 760 we have detected a similar pattern. Purification and details of the properties of cytochromes *c_L* and *c_H* of *M. organophilum* will be reported elsewhere.

The structure of PQQ has been elucidated (Salisbury *et al.*, 1979; Duine *et al.*, 1980) but its biosynthetic pathway is not yet known. Recent genetic studies in *A. calcoaceticus* (Goosen *et al.*, 1987) have shown that at least four different genes are involved in PQQ biosynthesis. There has so far been no such study in methylotrophic bacteria.

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

In *M. organophilum* strain XX, three regions of the genome containing several genes have been reported to be involved in the expression of an active MDH (Allen & Hanson, 1985), but the actual MDH structural gene was not located and possible defects in PQQ biosynthesis were not considered. The MDH structural gene from *Paracoccus denitrificans* has been cloned and its nucleotide sequence determined (Harms *et al.*, 1987). It corresponds to the *moxF* gene of *Methylobacterium* sp. AM1 described by Nunn & Lidstrom (1986). These authors isolated 26 mutants defective in methanol oxidation which could be classified with a genomic clone bank into 10 groups by complementation analysis (*mox* genes *A* to *H*). However, none of the *mox* mutants were involved in PQQ biosynthesis. In this paper, we report the isolation of the first PQQ mutant of *M. organophilum* and the localization of the gene involved, *pqqA*, next to the MDH structural gene *moxF*. The physiological behaviour of the PQQ mutant has already been reported (Biville *et al.*, 1988).

METHODS

Bacterial strains, phage and plasmids. These are listed in Table 1. Relevant characteristics of plasmids are shown in Fig. 1.

Media and growth conditions. *M. organophilum* was grown on minimal medium (MacLennan *et al.*, 1971) containing 0.5% (v/v) methanol or 0.2% (w/v) sodium succinate. LB medium (Miller, 1972) was used for *Escherichia coli* strains. Antibiotics, when added, were used at a final concentration of ($\mu\text{g ml}^{-1}$): kanamycin 25, ampicillin 100, rifampicin 20, tetracycline 10, chloramphenicol 25. PQQ, kindly provided by J. A. Duine (Delft University of Technology, The Netherlands), was used in aqueous solution after filter-sterilization. For growth of mutant MTM1, methanol medium was supplemented with PQQ (1 μM final concentration).

Biochemical techniques. For preparation of crude extracts, bacterial cells were suspended in 20 mM-potassium phosphate buffer pH 7.0 and passed twice through a French pressure cell at 110 MPa. After centrifugation for 30 min at 38000 g, the supernatant was used immediately for assays or frozen at -20°C . MDH was assayed according to Anthony & Zatman (1967) with 3 mM-KCN added to the cuvette. Specific activity was expressed as μmol dichlorophenolindophenol reduced min^{-1} (mg protein^{-1}). PQQ was detected in crude extracts with the apolcohol dehydrogenase from *Pseudomonas testosteroni* (Groen *et al.*, 1986) using a procedure described by Biville *et al.* (1988).

Electrophoresis in 10% (w/v) polyacrylamide gels was done according to the method of Laemmli (1970). After electrophoresis, proteins were stained with Coomassie blue R250 (Janvier & Gasser, 1987) and soluble cytochromes were revealed with 3,5,3',5'-tetramethylbenzidine according to Thomas *et al.* (1976).

Fig. 1. Relevant characteristics of the main plasmids and transposon used in this work.

Table 1. *Bacterial strains, phage and plasmids*

Bacterial strains	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
HB101	<i>hsdS20</i> (r_{B1}^- m_{B1}^-) <i>recAB proA2</i>	Boyer & Roulland-Dussoix (1969)
S17-1	Rec ⁻ , contains an RP4 derivative integrated in the chromosome providing <i>tra</i> functions used in this work for transfer of plasmids pSUP106, pRK290 and pLA2917	Simon <i>et al.</i> (1983)
S17-1 Rif ^r	Spontaneous rifampicin-resistant derivative of S17-1	This work
S17-1::Tn5	Insertion of Tn5 into the chromosome was achieved after infection with phage λ 467	This work
<i>Methylobacterium organophilum</i>		
DSM760	Wild-type strain	Deutsche Sammlung von Mikroorganismen
EMS mutants		
21111	MDH ⁻ (<i>moxF</i>)	This work
MTM1	PQQ ⁻ (<i>pqqA</i>)	This work
Tn5 insertion mutants		
MD4	Methanol ⁻ Cyt. c_{L}^+	This work
MD5	Methanol ⁺ Cyt. c_{L}^+	This work
MD14	Methanol ⁻ Cyt. c_{L}^-	This work
Kan ^r cartridge insertions		
MDQ1	PQQ ⁺ ; insertion near <i>pqqA</i>	This work
MDQ2	PQQ ⁻ ; insertion in <i>pqqA</i>	This work
Phage		
λ 467	<i>b221 cI857 rex::Tn5 O29 P80</i> ; a replication-deficient, integrationless phage used to deliver Tn5 into S17-1	Kleckner <i>et al.</i> (1977)
Plasmids		
pSUP106	Conjugative cosmid using RSF1010 origin of replication; Cmp ^r Tet ^r	Priefer <i>et al.</i> (1985)
pPM107	A pSUP106 derivative with substitution of the <i>Hind</i> III– <i>Bam</i> HI fragment of <i>tet</i> by the <i>Hind</i> III– <i>Bgl</i> II fragment of Tn5; Cmp ^r Kan ^r Ble ^r	This work
pPM111	A pBR322 derivative containing the whole central region of Tn5 and the <i>kan</i> promoter	Mazodier <i>et al.</i> (1983)
pRK290	IncP1, Tet ^r cloning vector with a single <i>Bgl</i> II cloning site	Ditta <i>et al.</i> (1980)
pLA2917	IncP, Tet ^r Kan ^r . A pRK290 derivative with a single <i>Bgl</i> II site affecting Kan ^r expression	Allen & Hanson (1985)
pMO340	17 kb of <i>M. organophilum mox</i> F region cloned in pSUP106	This work
pMO344	8 kb of <i>mox</i> F region cloned in pRK290 in one orientation	This work
R68-45	IncP1, Kan ^r Tet ^r Amp ^r with chromosome-mobilizing ability	Haas & Holloway (1976)
pJB3J1	A kanamycin-sensitive derivative of R68-45	Given by J. Beringer, University of Bristol, UK
R'51 to R'56; R'14	pJB3J1 derivatives containing DNA from <i>M. organophilum</i> with various Tn5 insertions	This work
pUC4K	A plasmid containing a 1400 bp DNA fragment encoding kanamycin resistance, with <i>Bam</i> HI and <i>Sal</i> I sites at each end to make an adaptable gene cartridge	Pharmacia (cat. no. 27-4987-01)
pMO500	6 kb of <i>M. organophilum pqqA</i> region cloned in pLA2917	This work
pMO512	2.5 kb of <i>pqqA</i> region cloned in pSUP106	This work
pMO5121	Same as pMO512, with Kan ^r cartridge near <i>pqqA</i>	This work
pMO5122	Same as pMO512, with Kan ^r cartridge in <i>pqqA</i>	This work
pNH3	A pEX2 derivative containing <i>mdh</i> , the structural gene of MDH from <i>Paracoccus denitrificans</i>	Harms <i>et al.</i> (1987)

Purification of MDH. The purification was carried out at 0–4 °C. Methanol (10 mM) was added as a stabilizer in all the buffers and solutions. Crude extract obtained from 15 g wet weight of bacterial cells was slowly acidified with 10% (v/v) acetic acid to pH 5.5. The precipitate was removed by centrifugation for 30 min at 20000 g. Protamine sulphate was slowly added with stirring to a final concentration of 0.3%. The resulting precipitate was discarded after centrifugation for 30 min at 20000 g. The supernatant was concentrated in an Amicon cell with a

YM30 membrane (molecular mass cut-off 30000 Da), diluted with ten volumes of 10 mM-acetate buffer pH 5.5 and concentrated again. This operation was repeated twice. The extract was applied to a column (20 × 2.5 cm) of CM-trisacryl (LKB-IBF), equilibrated with 10 mM-acetate pH 5.5. MDH was eluted with a linear gradient (0–150 mM) of NaCl in 10 mM-acetate buffer. The most active fractions were pooled, concentrated and applied to a Sephadex G200 column (1.5 × 60 cm). The active peak presented a single protein band after polyacrylamide gel electrophoresis (PAGE). The pure enzyme had a specific activity of 2.6 units. The purification procedure gave a 38% yield with a 12-fold purification of the enzyme. The enzyme was stored at –20 °C in 20 mM-phosphate buffer pH 7.0.

Immunological techniques. Antiserum was prepared in three-month-old virgin female Bouscat rabbits. Each animal received an intradermal injection of 30 µg pure MDH in 0.25 ml saline pH 7.0 emulsified in the same volume of incomplete Freund's adjuvant (Difco). One month later the rabbits were boosted with the same emulsion and bled 10 d later. Immunodiffusion experiments were done by the method of Ouchterlony as previously reported (Hontebeyrie & Gasser, 1975).

Molecular biology techniques. DNA isolation, endonuclease digestion, alkaline phosphatase treatment, ligation, DNA transformation, nick-translation and nitrocellulose filter hybridization were done essentially as described by Maniatis *et al.* (1982). Purification of *M. organophilum* total DNA was achieved essentially by the method of Marmur (1961).

Mutagenesis and mutant selection. Two millilitres of a culture of wild-type *M. organophilum* DSM 760 in methanol medium were treated during the early exponential phase (optical density, OD₆₀₀ = 0.6) with 0.01% (v/v) ethylmethane sulphonate (EMS). After 2 h incubation at 30 °C with shaking, the culture was centrifuged and washed twice in medium A without carbon source. The final pellet was resuspended in succinate minimal medium (4 ml) and the culture incubated at 30 °C for 24 h. For enrichment of mutants unable to grow on methanol, washed cells of the latter culture were used to inoculate (at OD₆₀₀ 0.05) methanol minimal medium (4 ml). At OD₆₀₀ 0.1, penicillin G (2000 IU ml⁻¹) was added and incubation was continued for another 24 h. The resulting culture was diluted and spread onto succinate minimal medium plates. Colonies were replica-plated onto succinate and methanol media and cells unable to grow on methanol medium were streaked for further purification.

Construction of the genomic bank. The genomic bank was constructed in the broad-host-range cosmid pSUP106, which contains a unique *Bam*HI cloning site in the *tet* gene (Fig. 1). Total DNA of *M. organophilum* DSM 760 was partially digested with *Sau*3A, ligated in a 3:1 ratio to plasmid pSUP106 cut by *Bam*HI and packaged *in vitro*. Bacteriophage λ packaging extracts were prepared and packaging was performed as described by Hohn & Collins (1980). The host strain *E. coli* S17-1 was infected and chloramphenicol-resistant, tetracycline-sensitive colonies were selected. *E. coli* S17-1 possesses plasmid-mobilization genes integrated within the chromosome and thus can be used for biparental crosses necessary to test genetic complementation of *M. organophilum* (Simon *et al.*, 1983).

Conjugation. In the absence of phage and of a transformation system, DNA could be introduced in *M. organophilum* only by conjugation. Conditions of conjugation between *E. coli* and *M. organophilum* (and reciprocal) have been described (Mazodier *et al.*, 1983).

Screening of the *M. organophilum* genomic bank constructed in *E. coli* was done by drop mating on minimal medium plates supplemented with 0.5% (v/v) methanol and 0.01% (w/v) sodium succinate. A recipient *M. organophilum* mutant was resuspended in LB medium and 25 drops were separately added to a plate; then 25 different *E. coli* S17-1 donor clones were patched, each to a different drop. Plates were then incubated for up to 4 d at 30 °C. The medium composition prevented growth of either donors or recipients but allowed the growth after complementation of the recipient mutation.

RESULTS

Selection of methanol oxidation mutants and screening of the genomic library built in pSUP106

Among 20 *M. organophilum* mutants unable to grow on methanol, two, 2111I and MTM1, were capable of normal growth on methylamine and consequently were not impaired in the serine pathway of formaldehyde incorporation. Their main biochemical characteristics are reported in Table 2 and Fig. 2. The mutant MTM1 can grow normally on methylamine, as in *M. organophilum* this compound is dissimilated through a methylglutamate dehydrogenase (Biville *et al.*, 1988), which is a flavohaemoprotein enzyme (Boulton *et al.*, 1980) rather than through methylamine dehydrogenase, a PQQ-dependent enzyme (Van der Meer *et al.*, 1987). This latter enzyme was not detected in this organism (Biville *et al.*, 1988), but it was present in *Methylobacterium* sp. AM1 (Eady & Large, 1968).

A genomic library of 800 clones was screened by conjugation with the two mutants 2111I and MTM1. No complementation of the PQQ⁻ mutant, MTM1, occurred. Complementation of mutant 2111I was observed with four *E. coli* clones. Restriction maps of the corresponding

Fig. 2. (a) PAGE and (b) immunoprecipitation in Ouchterlony plates of crude extracts from succinate-grown cells of *M. organophilum* DSM 760 wild-type (1) and of methanol oxidation mutants MTM1 (2), 2111I (3), MD4 (4), MD14 (5), MDQ2 (6) and MDQ1 (7). Lane 8 in (a) contains molecular mass markers. The amount of protein was 75 µg for each electrophoresis lane and in each well of the Ouchterlony plate. The central well received 80 µl of antiserum prepared against pure MDH.

Table 2. *Biochemical characteristics relevant to methanol oxidation in wild-type M. organophilum and in methanol oxidation mutants*

<i>M. organophilum</i> strain	MDH*			PQQ concn*† in:		Cytochromes <i>c</i>	
	Activity†	PAGE	Immuno-precipitation	Crude extracts	Culture supernatants	<i>c_L</i>	<i>c_H</i>
DSM 760 (wild-type)	0.51	+	+	1.15	0.33	+	+
EMS mutants							
2111I	0.02	+ w	—	0.06	1.0	+	+
MTM1	0	+ w	—	0.04	0	+	+
Chromosomal Tn5 mutants							
MD4	0.04	+	+	1.04	NT	+	+
MD14	0.03	+ w	+ w	0.09	2.0	—	+
Chromosomal cartridge mutants							
MDQ1	0.54	+	+	0.85	0.33	+	+
MDQ2	0	+ w	+ w	<0.02	0	+	+

w, Weak; NT, not tested.

* Succinate-grown cells, centrifuged in the early stationary phase.

† Units: µmol dichlorophenolindophenol reduced min⁻¹ (mg protein)⁻¹.

‡ Units: nmol PQQ (mg protein⁻¹) or (ml culture supernatant)⁻¹.

plasmids showed some common features. One of them, pMO340 (Fig. 3) was kept for further studies.

The mutation in strain 2111I affects the MDH structural gene

Plasmid pMO340 contains 17 kb of *M. organophilum* DNA. Subcloning was therefore necessary to locate the gene which could complement mutant 2111I. Plasmid pSUP106 does not

Fig. 3. Restriction maps of plasmids pMO340 and pMO344. The location and the direction of transcription of the structural gene of MDH, *moxF*, were established from the data shown in Fig. 4. Numbered triangles in pMO340 indicate Tn5 insertions.

allow stable complementation (see below). In order to obtain true complementation of the mutation in strain 2111I, a *Bgl*II digestion of pMO340 was cloned at the *Bgl*II site of the replicative plasmid pRK290. The resulting plasmids were introduced into *E. coli* S17-1 by transformation and transferred to mutant 2111I by conjugation. Recipient selection was achieved on methanol medium containing tetracycline. Plasmid pMO344, containing an 8 kb fragment (Fig. 3) complemented mutant 2111I.

In order to identify the gene complementing the mutation in strain 2111I, two fragments of pNH3, which encodes the MDH structural protein of *Paracoccus denitrificans* (Harms *et al.*, 1987), were used as hybridization probes (Fig. 4). A 1.4 kb *Sal*I–*Kpn*I fragment of plasmid pMO340 showed cross-hybridization to the MDH structural gene of *P. denitrificans*. In accordance with the nomenclature of Nunn & Lidstrom (1986) we refer to the homologous gene of *M. organophilum* as *moxF*.

Behaviour of plasmid pSUP106 in M. organophilum

The conjugative cosmid pSUP106 was designed to allow the transfer of large DNA fragments to a wide range of Gram-negative bacteria (Priefer *et al.*, 1985). Therefore, it was a potentially good candidate to establish a possible genetic link between the MDH and PQQ genes, provided that it could easily be transferred to and from *M. organophilum* and maintained in this organism to ensure *trans* complementation.

pSUP106 contains resistance genes to chloramphenicol and tetracycline. *M. organophilum* has a high level of natural resistance to chloramphenicol and therefore this antibiotic cannot be used as a selectable marker. On the other hand, *M. organophilum* is sensitive to low concentrations of tetracycline. However, after crosses of *E. coli* S17-1(pSUP106) × *M. organophilum* DSM 760 no tetracycline-resistant exconjugant could be isolated on methanol medium. This might be explained by an absence of transfer of pSUP106, by the inability of this plasmid to replicate in *M. organophilum*, or by poor expression of the *tet* gene.

We have previously shown that the neomycin-resistance gene of Tn5 is highly expressed in *M. organophilum* (Mazodier *et al.*, 1982). Therefore, the *tet* gene of pSUP106 was replaced by a fragment of the central region of Tn5 containing the kanamycin-resistance gene and its promoter (plasmid pPM107, Table 1). After conjugation between *E. coli* S17-1(pPM107) and *M. organophilum* DSM 760, selection was made on minimal medium containing methanol and kanamycin. The undiluted mating mixture led to a heavy background of pink growth of *M. organophilum* but no single colonies could be obtained when dilutions were plated. Attempts

Fig. 4. Hybridization of restriction fragments of pMO340 with plasmid pNH3, which encodes the MDH structural gene from *Paracoccus denitrificans* (Harms *et al.*, 1987). After digestion by *Eco*RI and *Sal*I, two fragments of pNH3 were electroeluted and used as probes: probe 1 was the 1.4 kb *Eco*RI fragment which corresponds to the 5' end of the MDH structural gene and probe 2 was the 0.8 kb *Eco*RI-*Sal*I fragment of the 3' end of the gene. Plasmid pMO340 was digested with *Eco*RI, *Sal*I and *Sal*I/*Kpn*I, analysed on agarose gel (left) and hybridized with the two probes. The pattern of hybridization allowed the location of the MDH structural gene and its orientation leftward as shown in Fig. 3 to be determined.

to obtain single colonies from the background growth were not successful. To check that the background growth was the consequence of plasmid transfer and was not due to protection by the kanamycin-resistance gene present in the donor *E. coli*, the latter gene was introduced into the non-conjugative plasmid pBR322 (giving plasmid pPM111, Table 1). After crosses of *E. coli* S17-1(pPM111) $\times$ *M. organophilum* DSM 760, no background growth was observed on methanol medium complemented with kanamycin.

The conclusion is that pSUP106 is probably transferred to *M. organophilum* but cannot replicate in this environment. The background growth can be explained by a high level of transfer of pSUP106 and a subsequent burst of expression of the kanamycin-resistance gene, followed by a decrease of resistance by a dilution effect at each cell division. If this hypothesis is correct, pSUP106 containing cloned *M. organophilum* DNA could be used as a suicide plasmid producing transient complementation, possibly followed by recombination between homologous cloned and chromosomal DNA, thus permitting the introduction of modifications into the genome of *M. organophilum*. This hypothesis is confirmed in the following section.

Table 3. *Specificity of partial chromosome exchange in M. organophilum as evidenced by growth on methanol medium of Tn5 insertion mutants after integration of pMO340 plasmids containing different Tn5 insertions*

Plasmid in donor strain (<i>E. coli</i> S17-1)	Growth on methanol (medium of Tn5-containing recipient mutants)	
	Insertion no. 4 (strain MD4)	Insertion no. 14 (strain MD14)
pMO340	+	+
pMO340::Tn5 no. 4	—	+
pMO340::Tn5 no. 14	+	—
pMO340::Tn5 no. 5	+	+
pSUP106 (control)	—	—

Directed insertions of transposon Tn5 into the M. organophilum genome

The transposon Tn5 was first randomly inserted into various sites of the plasmid pMO340. For this, pMO340 was introduced into *E. coli* S17-1::Tn5 (Table 1) by transformation. Selection of various pMO340::Tn5 insertion derivatives was achieved by a cross *E. coli* S17-1::Tn5(pMO340) × *E. coli* S17-1 Rif^r and subsequent selection of Rif^rCmp^rKan^r exconjugants.

In order to introduce Tn5 into the genome of *M. organophilum*, 26 clones of *E. coli* S17-1 Rif^r(pMO340::Tn5), numbered 1 to 26, were crossed with *M. organophilum* wild-type. *M. organophilum* exconjugants were selected on succinate medium containing kanamycin. Only six donor clones had the ability to give stable kanamycin-resistant *M. organophilum* exconjugants, which appeared as large colonies on a background of microcolonies. After each cross, seven colonies of Kan^r exconjugants were re-isolated and examined for methanol utilization. None of the *M. organophilum* exconjugant clones (7/7) which received Tn5 insertions 4 or 14 grew on methanol medium. Those which received Tn5 insertions 5, 23 or 25 were unaffected in methanol utilization. Restriction mapping of the corresponding plasmids gave the locations of the different insertions of Tn5 in pMO340 (Fig. 3).

M. organophilum exconjugants with Tn5 insertions 4 and 14, which affected regions involved in methanol utilization, were named mutants MD4 and MD14 respectively. Their biochemical characteristics are given in Table 2. Spontaneous reversion of these mutants to the wild-type phenotype, i.e. growth on methanol medium, occurred at a frequency of about 10⁻⁹, with simultaneous loss of kanamycin resistance.

The specificity of the insertions of Tn5 into the genome of *M. organophilum* was checked by crosses of mutants MD4 and MD14 with *E. coli* S17-1(pMO340) and S17-1(pMO340::Tn5) (numbers 4, 5 and 14). The results of these conjugations (Table 3) exclude the possibility of Tn5 transposition from pSUP106 to the chromosome of *M. organophilum*. A complete lack of Tn5 transposition in *M. organophilum* has been observed previously (Mazodier *et al.*, 1983). The fact that the mutations in strains MD4 and MD14 can be abolished in this way suggests rather that Tn5 was integrated as a part of the DNA of pMO340::Tn5 by a double crossing-over between homologous sequences present both in pMO340 and in the chromosome of *M. organophilum*. The fraction of pMO340 originating from pSUP106 was not integrated in the chromosome of *M. organophilum*: in 'dot-blot' analyses of the genomes of MD4, MD5 and MD14, no hybridization was observed when pSUP106 was used as a probe, but a strong signal was obtained with an internal Tn5 DNA fragment as probe (results not shown).

In another experiment the stability of Tn5 insertion in pMO340 was checked by crosses between *E. coli* S17-1(pMO340::Tn5 no. 5) and *M. organophilum* mutant 2111I, followed by selection on succinate medium containing kanamycin. Of 34 colonies obtained on succinate medium, 33 could also grow on methanol medium. This result also suggested the proximity of Tn5 insertion no. 5 and the mutation present in 2111I.

In conclusion, the behaviour of pSUP106 as a suicide plasmid in *M. organophilum* allows the possibility of creating insertion mutations in defined regions of the chromosome of this organism.

Cloning the moxF region on R' plasmids

In an attempt to locate the mutation of strain MTM1 next to the *moxF* region, a technique employing R' plasmids was used. Moore *et al.* (1983) cloned up to 200 kb of chromosomal DNA from *Methylophilus methylotrophus* in R68-45, a plasmid able to mobilize efficiently the chromosome of several Gram-negative bacteria. As a chromosome-mobilizing vector, the plasmid pJB3J1, a kanamycin-sensitive mutant of R68-45, was used to allow the use of the kanamycin resistance of Tn5 as a chromosomal marker. *M. organophilum* mutant MD5, which has a Tn5 insertion proximal to the *moxF* region (Fig. 3), received pJB3J1 from *E. coli*. After selection for tetracycline-resistant exconjugants and subsequent purifications, a clone of *M. organophilum* MD5(pJB3J1) was conjugated with *E. coli* HB101 and kanamycin-resistant *E. coli* exconjugants were selected. Six purified colonies, called HB101(R'51) to HB101(R'56) were crossed with mutants 2111I, MD4, MD14 and MTM1. All four mutants were complemented by all the R' plasmids except for one, R'56, which failed to complement mutant MTM1.

These results indicated that the piece of *M. organophilum* DNA present in these plasmids was, as expected, contiguous to Tn5 insertion no. 5. It was larger than the insert in pMO340 because it complemented the distal mutation in MTM1. Among other R' plasmids built on the same model, R'14B, the smallest able to complement both 2111I and MTM1, had an insert of about 30 kb, demonstrating that at least one PQQ biosynthesis gene is in the vicinity of the *moxF* region. This gene was termed *pqqA*. Most of the R' plasmids were very stable in *E. coli*, where they could be maintained for months.

Subcloning of pqqA in pLA2917

Plasmid pLA2917 is a derivative of pRK290, able to replicate in *M. organophilum* (Fig. 1, Table 1). Plasmid R'51 DNA was partially digested with *Sau3A* and ligated to pLA2917 cut by *Bgl*II. After transformation in *E. coli* S17-1, tetracycline-resistant, kanamycin-sensitive clones were selected. Several clones could effect complementation of the mutation in strain MTM1. Plasmid pMO500, with an insertion of 6 kb of *M. organophilum* DNA, was isolated from one of these clones (Fig. 5).

Directed mutagenesis of the chromosomal pqqA region

MTM1 was isolated after mutagenesis with EMS, a mutagen known to produce multiple, tightly grouped mutations. To exclude any additional undetected PQQ mutations which might be present in the chromosome of strain MTM1 we decided to replace the *pqqA* chromosomal region of wild-type *M. organophilum* by an homologous plasmidic region containing an insertion with a resistance marker, using the procedure of gene substitution by double crossing-over described for pMO340::Tn5 in this work.

Different restriction fragments of pMO500 were subcloned in pSUP106 to locate more precisely the gene *pqqA* involved in complementation of the mutation in strain MTM1. The *pqqA* gene was located in the 2.5 kb *Bgl*II-*Xho*I DNA fragment of plasmid pMO512 (Fig. 5).

The Kan^r cartridge of pUC4K (Table 1) was cloned into two different restriction sites of pMO512, *Bam*HI and *Sal*I, yielding plasmids pMO5121 and pMO5122 respectively (Fig. 5). After transformation in *E. coli* S17-1 and subsequent transfer in *M. organophilum*, exconjugants were selected for kanamycin resistance and tested for growth on methanol medium. The Kan^r exconjugant MDQ1, selected after the cross using pMO5121, was not affected in growth on methanol. With pMO5122 the kanamycin-resistant exconjugant, MDQ2, was unable to synthesize PQQ (Table 2) or grow on methanol medium without added PQQ. MDQ2 could be complemented by pMO500. Accordingly *pqqA* was located in the 1.5 kb *Bgl*II-*Bam*HI fragment. The biochemical characteristics of strains MDQ1 and MDQ2 are given in Table 2.

Fig. 5. Restriction map of plasmids containing DNA of the *pqqA* region from *M. organophilum*. Plasmid pMO512 complemented the mutation MTM1. The activity of the *pqqA* gene was affected by the Kan^r cartridge inserted at the *SalI* site but not by an insertion at the *BamHI* site.

DISCUSSION

Plasmid pSUP106 was designed on the basis of conventional *E. coli* vectors and modified to include mobilization and broad-host-range replication functions of the IncQ plasmid RSF1010 (Priefer *et al.*, 1985). Stable replication of pSUP106 has been obtained in various Gram-negative genera such as *Rhizobium*, *Agrobacterium* and *Pseudomonas*.

We have observed the inability of pSUP106 and of its cosmid hybrids to replicate in *M. organophilum*. This property made it possible to use pSUP106 hybrids as suicide vectors and therefore, after appropriate Tn5 insertions, to proceed to localized mutagenesis in the chromosome of *M. organophilum*. Thus all possible multiple mutations so frequently found after chemical mutagenesis were excluded. Moreover, DNA exchange between homologous chromosomal and plasmidic regions adjacent to a Tn5 insertion allowed the modification of the genome, an important feature for strain improvement. For example, the wild-type MDH structural gene might be replaced by an *in vitro*-modified gene cloned in pMO340::Tn5 no. 5, simply by selecting kanamycin-resistant colonies of cells containing this Tn5 insertion, which is close to the MDH structural gene.

Finally, taking advantage of the introduction of precisely localized chromosomal markers, prime plasmids were constructed including large fragments of chromosomal DNA from *M. organophilum*. With these prime plasmids, the *pqqA* gene has been located in the vicinity (≤ 30 kb) of the *moxF* region.

The *pqqA* gene was not found in the gene library built in pSUP106. This gene was subcloned from the plasmid R'51 and labelled with two kanamycin resistance gene insertions, one inside *pqqA* (strain MDQ2) and the other next to *pqqA* (MDQ1) (Fig. 5). This will allow further easy cloning of the *pqqA* region and of the DNA fragment between *pqqA* and *moxF*. The mutagenesis by Tn5 of R'51 DNA fragments inserted in pSUP106 and subsequent transfer to the *M. organophilum* chromosome will permit further localized mutagenesis of this region of the genome. Indeed, several other *pqq* genes remain to be found. At least four genes have been

shown to be involved in PQQ biosynthesis in *Acinetobacter calcoaceticus* (Goosen *et al.*, 1987) and they have to be located in *M. organophilum*.

Allen & Hanson (1985) reported a similar location of the defect of our mutant 2111I to that found in this work and we have shown that this mutation affects the structural gene of MDH (*moxF*). The Tn5 insertion no. 14 present in mutant MD14 was located in the close vicinity of *moxF*. This insertion affects the synthesis of cytochrome c_L . A similar clustering of genes is also present in *Methylobacterium* sp. AM1 (*moxF* and *moxG*, Nunn & Lidstrom, 1986), even though these two *Methylobacterium* species share only 29% DNA homology (Hood *et al.*, 1987).

The results reported in Table 2 show that the amount of PQQ in crude extracts is very low when the apo-MDH, i.e. the product of the structural gene, is not synthesized. However, under these conditions, when the synthesis of PQQ is unaffected, an excess of this compound was found in the culture supernatant, as compared with the wild-type. This observation confirmed that free PQQ cannot accumulate in the cell, as has already been observed (Biville *et al.*, 1988). On the other hand, a very low amount of apo-MDH was present in crude extracts of mutants MTM1 and MDQ2, unable to synthesize PQQ (Table 2). It is not possible to decide whether PQQ has a positive effect on the biosynthesis of apo-MDH or a stabilizing effect on an unstable apoenzyme. The latter hypothesis is more probable since generally prosthetic groups are not considered as inducers of their own apoenzymes.

The mutation of strain MD14 was located in a region apparently coding for cytochrome c_L synthesis and downstream of the gene *moxF* encoding apo-MDH. Notwithstanding this location the amount of apo-MDH detected in extracts of MD14 was very low (Fig. 2) and, as already discussed, PQQ was detected mainly in the culture supernatant (Table 2). This observation suggests that the assembly of holo-MDH of *M. organophilum* cannot be carried out correctly in the absence of cytochrome c_L . At present, the observed phenotype of mutant MD4 is not clearly explicable. Although MDH and cytochrome c_L were apparently normal, in accordance with the location of the mutation, MDH activity was practically absent in crude extracts. A possible hypothesis is the presence of a positive effector associated with MDH activity, which might be encoded in this region of the genome.

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