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# Isolation, Phenotypic Characterization, and Complementation Analysis of Mutants of *Methylobacterium extorquens* AM1 Unable To Synthesize Pyrroloquinoline Quinone and Sequences of *pqqD*, *pqqG*, and *pqqC*

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**Aerobic gram-negative methylotrophs oxidize methanol to formaldehyde by using a methanol dehydrogenase that has pyrroloquinoline quinone (PQQ) as a prosthetic group. Seventy-two mutants which are unable to grow on methanol unless the growth medium is supplemented with PQQ have been isolated in the facultative methanol utilizer *Methylobacterium extorquens* AM1. In addition, 12 previously isolated methanol oxidation mutants of *M. extorquens* AM1 were shown to be able to grow on methanol in the presence of PQQ. These putative PQQ biosynthesis mutants have been complemented by using previously isolated clones containing *M. extorquens* AM1 DNA, which were known to contain genes necessary for oxidation of methanol to formaldehyde (*mox* genes). Subcloning and transposon mutagenesis experiments have assigned these mutants to five complementation groups in two gene clusters. Representatives of each complementation group were shown to lack detectable PQQ in the growth medium and in cell extracts and to contain methanol dehydrogenase polypeptides that were inactive. Therefore, these mutants all appear to be defective in PQQ biosynthesis. PQQ biosynthesis mutants of *Methylobacterium organophilum* DSM 760 and *M. organophilum* XX were complemented by using *M. extorquens* AM1 subclones, and PQQ biosynthesis mutants of *M. extorquens* AM1 and *M. organophilum* XX were complemented by using *M. organophilum* DSM 760 subclones. This analysis suggested that a total of six PQQ biosynthesis complementation groups were present in *M. extorquens* AM1 and *M. organophilum* DSM 760. A 2-kb *M. extorquens* AM1 DNA fragment that complemented the MoxO class of PQQ biosynthesis mutants was sequenced and found to contain two complete open reading frames and the N-terminal sequence of a third. These genes, designated *pqqDGC*, had predicted gene products with substantial similarity to the gene products of corresponding *pqq* genes in *Acinetobacter calcoaceticus* and *Klebsiella pneumoniae*. *pqqD* encodes a 29-amino-acid peptide which contains a tyrosine residue and glutamate residue that are conserved in the equivalent peptides of *K. pneumoniae*, PqqA (23 amino acids), and *A. calcoaceticus*, PqqIV (24 amino acids), and are thought to be the precursors for PQQ biosynthesis. The organizations of a cluster of five PQQ biosynthetic genes appear to be similar in four different bacteria (*M. extorquens* AM1, *M. organophilum* DSM 760, *K. pneumoniae*, and *A. calcoaceticus*). Our results show that a total of seven *pqq* genes are present in *M. extorquens* AM1, and these have been designated *pqqDGCBA* and *pqqEF*.**

Gram-negative aerobic methylotrophic bacteria that grow on methanol use methanol dehydrogenase (MDH) to oxidize methanol to formaldehyde, which may then be further oxidized or assimilated into cell carbon (27). MDH is a tetrameric enzyme located in the periplasm that contains pyrroloquinoline quinone (PQQ) as the prosthetic group and also contains $Ca^{2+}$ (37, 40). PQQ was first identified as the prosthetic group of MDH and is now also known to be the prosthetic group of a few other bacterial dehydrogenases that oxidize alcohols or sugars (9). The biosynthetic pathway of PQQ has not yet been determined, but the biosynthetic precursors are known to be tyrosine and glutamate (19).

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Mutants unable to produce PQQ have been reported in *Acinetobacter calcoaceticus* (14), *Methylobacterium organophilum* DSM 760 (5, 6), *Klebsiella aerogenes* (34), and *Methylobacillus flagellatum* (13). In *A. calcoaceticus* (14) and *K. pneumoniae* (34), *pqq* genes have been isolated and sequenced. In *A. calcoaceticus*, four *pqq* genes (I, II, III, and IV) were mapped in a 5,085-bp DNA fragment in the order IV-I-II-III. Between *pqqI* and *pqqIV*, an additional open reading frame (*pqqV*), not required for PQQ biosynthesis, is present (14). In *K. pneumoniae*, six genes were identified in the following order: *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE*, *pqqF* (34). Substantial similarities were observed between *pqqIV* and *pqqA*, *pqqV* and *pqqB*, *pqqI* and *pqqC*, *pqqII* and *pqqD*, and *pqqIII* and *pqqE*. In addition, a partial open reading frame (*orfX*) identified upstream of *pqqA* showed similarity to a partial open reading frame (*orfR*) located downstream of gene III (34). *pqqIV* and *pqqA* both encode small peptides that contain conserved tyrosine and glutamate residues. This peptide has been proposed to be the starting material for PQQ (14, 34). It was observed that *pqqF* contains sequence similarity to a family of

proteases and that *orfX* and *orfR* show similarity to a human dipeptidase (34). It is possible that these gene products are involved in processing of the peptide during PQQ biosynthesis (34).

Six Pqq complementation groups have also been identified in *M. organophilum* DSM 760 and were assumed to correspond to genes designated *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE*, and *pqqF* (6). Sequence data are not yet available for these genes, and their correspondence to the genes identified in *K. pneumoniae* and *A. calcoaceticus* is not known. The *pqq* designations of these genes were made before the *pqq* genes of *K. pneumoniae* were isolated and sequenced, and so they bear no known relationship to each other.

In *Methylobacterium extorquens* AM1, a large number of complementation groups have been identified in mutants defective in the oxidation of methanol to formaldehyde (26). In some cases, sequence and expression data have confirmed the presence of genes equivalent to these complementation groups. These include four clustered genes (*moxFJGI*) that encode the 60-kDa MDH alpha subunit (*moxF*), the 10-kDa MDH beta subunit (*moxI*), the 21-kDa MDH-specific cytochrome $c_L$ (*moxG*), and a polypeptide of 30 kDa with unknown function (*moxJ*) (2, 38, 39). A fifth *mox* gene (*moxR*) has been identified 3′ to the *moxFJGI* cluster in *Paracoccus denitrificans*, and sequence homology suggests that this gene is also present in the same position in *M. extorquens* AM1 (45).

Three other *M. extorquens* AM1 complementation groups (MoxA, MoxK, and MoxL) (38, 39) define a gene cluster 3′ to the *moxFJGIR* cluster that is involved in providing calcium to MDH (40). Six other Mox complementation groups (MoxM, MoxN, MoxD, MoxQ, MoxE, and MoxB) that are required for transcription of *mox* genes have been identified in *M. extorquens* AM1 (36, 46). In *P. denitrificans*, three additional genes, *moxXYZ*, that are required for transcription of *mox* genes have been identified by sequencing and mutation (16). Two other groups of Mox mutants (MoxC and MoxH) whose function was unknown were identified (38, 39). In this study, we show that these groups are involved in PQQ biosynthesis (see below).

In this study, we describe the characterization of 72 new *M. extorquens* AM1 mutants and 14 previously isolated *M. extorquens* AM1 and *M. organophilum* XX mutants, all unable to synthesize PQQ. The complementation patterns and phenotypic characteristics of the mutants in *M. extorquens* AM1 have been determined, and their correspondence to complementation groups of *M. organophilum* DSM 760 has been established. In addition, we have sequenced a 2,049-bp *Bgl*II-*Hin*dIII DNA fragment which complemented mutants in the *M. extorquens* AM1 MoxO class and have identified three *pqq* genes (*pqqDGC*) in this region.

## MATERIALS AND METHODS

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

**Media and growth conditions.** *M. extorquens* AM1 and *M. organophilum* DSM 760 were grown on the minimal medium (medium A) of MacLennan et al. (31) or the minimal medium described previously (12), containing 0.5% (vol/vol) methanol, 0.2% (wt/vol) methylamine hydrochloride, or 0.2% (wt/vol) succinate. When required, filter-sterilized PQQ (Methoxatin; Fluka) was added to 1 μM. *Escherichia coli* strains were grown on LB medium (35). Antibiotics were added at the following final concentrations (micrograms per milliliter): tetracycline, 10; kanamycin, 25 (*M. extorquens* AM1) or 50 (*E. coli*); ampicillin, 100; and streptomycin, 10. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to media at 40 μg/ml.

**Mutagenesis.** Rifamycin-resistant *M. extorquens* AM1 (38) was mutagenized with ethyl methanesulfonate (EMS) as follows. A 1-ml aliquot of mid-log-phase cells was washed and resuspended in sterile medium, and 15 μl of EMS was added. The cells were incubated with shaking at 30°C for 2 h. The cells were then washed twice in sterile medium and resuspended in 1 ml of sterile medium, and aliquots were plated onto selective plates of nutrient agar (Difco, Detroit, Mich.) supplemented with 0.2% (vol/vol) methanol and 0.05% (vol/vol) allyl alcohol (Aldrich Chemical, Milwaukee, Wis.). This treatment resulted in approximately 1% survival when mutagenized cells were plated onto nutrient agar plates and compared with plate counts made from the culture just prior to EMS addition.

**Transposon mutagenesis.** Tn*5lac* insertions were generated in cloned *M. extorquens* AM1 DNA in *E. coli*, using P1-Tn*5lac* as described by Kroos and Kaiser (24). Tn*phoA* insertions were generated in cloned *M. extorquens* AM1 DNA in *Salmonella typhimurium* by using P22-Mu d::Tn*phoA* (41) by the method described by Hughes and Roth (20). In each case, pools of mutagenized plasmids were isolated and used to transform *E. coli*, selecting for the kanamycin resistance marker in Tn*phoA* or Tn*5lac*. Kanamycin-resistant colonies were pooled and used as donors in triparental matings with *M. extorquens* AM1 as the recipient, again selecting for kanamycin resistance. Individual colonies (100 to 200 in each case) were picked and used for complementation and mapping studies. *M. extorquens* AM1 strains containing plasmids with Tn*phoA* insertions were screened for alkaline phosphatase activity on minimal methanol medium (12) containing 0.04 mg of 5-bromo-4-chloro-3-indolylphosphate per ml. Neither controls with the same plasmids lacking Tn*phoA* nor the test strains showed evidence of activity.

**Bacterial matings.** Biparental matings with *E. coli* strains containing pLA2917 derivatives were performed as previously described (6). With *E. coli* strains harboring pVK100 derivatives, triparental matings were performed with the mobilizing plasmid pRK2013 as previously described (38). Mating mixtures were plated on succinate, methanol, or methylamine minimal medium when *Methylobacterium* strains were recipients or on LB agar when *E. coli* strains were recipients. In each case, the appropriate antibiotics were included.

**DNA manipulations.** Plasmid DNA from *E. coli* strains was prepared as described by Maniatis et al. (32) and by either the method of Humphrey et al. (21) or the method of Ish-Horowicz and Burke (22) for large-scale plasmid preparations. Endonuclease digestions and ligations were done as recommended by the manufacturer. DNA transformation in *E. coli* was performed as described by Maniatis et al. (32). Agarose gel electrophoresis was carried out in 0.8% gels with Tris-borate running buffer in a horizontal electrophoresis apparatus. DNA sequencing was done according to the dideoxy-chain termination method of Sanger et al. (42), using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio), or by the UCLA Sequencing Facility on an Applied Biosystems model 373A automated sequencer.

**Biochemical techniques.** MDH activity was assayed by the phenazine methosulfate-dichlorophenol indolphenol method described by Anthony and Zatman (3). PQQ was assayed as previously described (5), using the apoethanol dehydrogenase purified from *Pseudomonas testosteroni* (15). Immunoblotting of protein gels with antisera against the alpha and beta subunits of MDH and cytochrome $c_L$ was carried out as described previously (39). Protein was estimated by the method of Lowry et al. (29).

TABLE 1. Bacterial strains, plasmids, and phages used in this study

| Strain, phage, plasmid, or clone | Relevant trait(s) | Source or reference |
|---|---|---|
| *E. coli* | | |
| DH5α | $r^- m^+$ *recA1 lacZYA* ϕ80 d*lac*Δ(*lacZ*)*M15* | Bethesda Research Laboratories, Inc. |
| HB101 | *recA* $Str^r$ | 8 |
| SF800 | $Nal^r$ *polA* | 17 |
| S17.1 | *recA thi pro* | 43 |
| *M. extorquens* AM1 $Rif^r$ mutants | | 38 |
| UV45 | MoxC | 38 |
| EMS12 | MoxT | This study |
| UV46 | MoxO | 38 |
| UV48 | MoxH | 38 |
| EMS7.42 | MoxU | This study |
| *M. organophilum* XX mutants | | |
| PT34 and PT47 | Group VID (MoxP) | 30 |
| *M. organophilum* DSM 760 mutants | | Deutsche Sammlung von Mikro-organismen |
| MTM1 | *pqqA* | 33 |
| 452 | *pqqB* | 6 |
| 474 | *pqqC* | 6 |
| 71 | *pqqD* | 6 |
| 430 | *pqqE* | 6 |
| 530 | *pqqF* | 6 |
| *P. testosteroni* | ATCC 15667 (wild type) | 15 |
| Phages | | |
| P1::Tn*5lac* | P1 phage, Tn*5lac* | 24 |
| P22-Mu d::Tn*phoA* | P22-Mu d phage, Tn*phoA* | 41 |
| Plasmids | | |
| pVK100 | $Tc^r$, $Km^r$ IncP1 cosmid | 23 |
| pLA2917 | $Tc^r$, $Km^r$ IncP1 cosmid | 1 |
| pRK2013 | $Km^r$ mobilizing helper | 11 |
| pRK2073 | $Sm^r$ mobilizing helper | 11 |
| pRK310 | $Tc^r$ IncPI | 10 |
| pUC18/19 | $Ap^r$ *lacZ'*, multiple cloning site | 47 |
| *M. organophilum* DSM 760 clones | | |
| pMO550 | pLA2917 containing *pqqABCD* | 6 |
| pMO552 | pLA2917 containing *pqqABC* | 6 |
| pMO551 | pLA2917 containing *pqqCD* | 6 |
| pMO500 | pLA2917 containing *pqqA* | 6 |
| pMO230 | pLA2917 containing *pqqE* | 6 |
| pMO611 | pLA2917 containing *pqqF* | 6 |
| *M. extorquens* AM1 clones | | |
| pEL41 | pVK100 containing a 24-kb *Hin*dIII fragment (MoxPCVTO) | 38 |
| p1130 | pRK310 containing a 10.3-kb *Pst*I fragment (MoxCVTO) | 38 |
| p1130C | pRK310 containing a 3.4-kb *Pst*I-*Hin*dIII fragment (MoxPC) | This study |
| p1130C::Tn*5*-6 | p1130C containing Tn*5lac* for MoxP | This study |
| p1130C::Tn*5*-82 | p1130C containing Tn*5lac* for MoxC | This study |
| p1130C-PBg | pRK310 containing a 1.8-kb *Pst*I-*Bgl*II fragment | This study |
| p1130C-BgH | pRK310 containing a 1.6-kb *Bgl*II-*Hin*dIII fragment | This study |
| p1130D | pRK310 containing a 6.8-kb *Hin*dIII-*Pst*I fragment (MoxO) | This study |
| p1130D::Tn*5*-136 | p1130D containing a Tn*5lac* insertion for MoxO | This study |
| p1130D::Tn*5*-19 | p1130D containing a Tn*5lac* insertion for MoxO | This study |
| p1130D-HBg | pRK310 containing a 1.7-kb *Hin*dIII-*Bgl*II fragment (MoxO) | This study |
| p1130D-HBg2.1 | pRK310 containing a 2.1-kb *Hin*dIII-*Bgl*II fragment (MoxO) | This study |
| pDN48C-46 | pVK100 containing a 19.6-kb *Hin*dIII fragment (MoxHU) | 38 |
| pELH2 | pRK310 containing a 4-kb *Hin*dIII fragment of p48C-46 (MoxHU) | This study |
| pELH2::Tn*phoA*-E1 | pELH2 containing Tn*phoA* in MoxH | This study |
| pCM187 | $Ap^r$, 2.1-kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg2.1 in pUC18 | This study |
| pCM188 | $Ap^r$, 1.7-kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg in pUC18 | This study |
| pCM198 | $Ap^r$, 1.7-kb *Hin*dIII-*Eco*RI subclone from p1130D-HBg in pUC19 | This study |

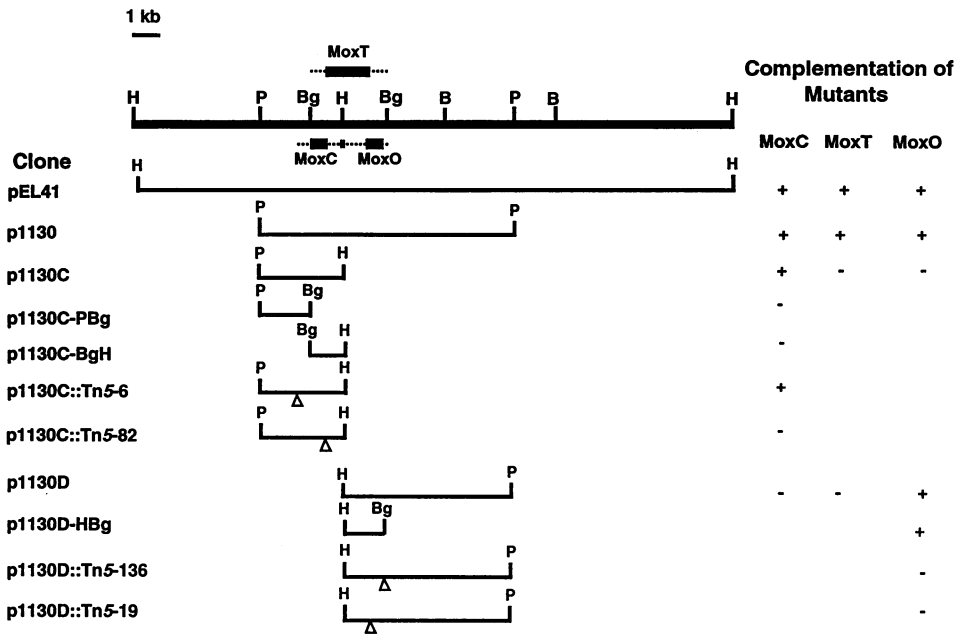


FIG. 1. Restriction map and complementation analysis of MoxC, MoxT, and MoxO mutants of *M. extorquens* AM1, using pEL41 and its derivatives. Filled boxes represent minimum gene sizes; dotted lines represent maximum sizes; triangles denote Tn*5lac* insertions. H, *Hin*dIII; P, *Pst*I; Bg, *Bgl*II; B, *Bam*HI.

**Nucleotide sequence accession number.** The nucleotide sequence of the *pqqDGC* region reported here has been assigned GenBank accession number L25889.

## RESULTS

**Selection of Mox mutants impaired in PQQ biosynthesis.** *M. extorquens* AM1 cells were mutagenized as described in Materials and Methods and plated on allyl alcohol plates to select for mutants unable to oxidize methanol to formaldehyde. Those colonies that grew on allyl alcohol plates were tested for the ability to grow on methanol and methylamine. A total of 214 mutants unable to grow on methanol but still capable of growth on methylamine were selected and were presumed to be Mox mutants. Of these, 72 were able to grow on methanol in the presence of PQQ and were presumed to be deficient in PQQ biosynthesis. Analysis of the other Mox mutants will be described elsewhere. In addition, the previously described Mox mutants of *M. extorquens* AM1 (38, 39) and a set of previously described *M. organophilum* XX Mox mutants (30) were tested for growth on methanol in the presence of PQQ. Fourteen of these mutants were positive for growth, suggesting they were also PQQ biosynthesis mutants. For *M. extorquens* AM1, these included the MoxC mutants AA18, AA40, UV40, UV41, UV42, UV44, UV45, UV47, UV50, and UV52, the MoxH mutant UV48, and the MoxD mutant UV46. UV46 had previously been shown to be in a separate complementation group from the other two MoxD mutants (4). These other MoxD mutants do not grow on methanol in the presence of PQQ. Only one *M. organophilum* XX group (group VID) was positive for growth and included mutants PT34 and PT47.

**Complementation of *M. extorquens* AM1 PQQ biosynthesis mutants with *M. extorquens* AM1 DNA.** Each of the putative *M. extorquens* AM1 PQQ biosynthesis mutants was tested for complementation by previously isolated *M. extorquens* AM1 clones that were known to contain *mox* genes (38, 39). These included the clones previously designated HIND-AB, HIND-CD, HIND-E, HIND-FG, and HIND-H, each containing an *M. extorquens* AM1 *Hin*dIII insert of 8 to 20 kb (38, 39). Two of these clones, HIND-CD (pEL41) and HIND-H (pDN48-46C), complemented all 72 new PQQ biosynthesis mutants as well as the 12 previously described *M. extorquens* AM1 mutants. These clones were chosen for further studies, and a variety of subclones and transposon insertions were generated for each of them. Further analysis allowed us to place the 84 mutants into five complementation groups (see below).

**C, T, and O complementation groups.** Fifty of the new PQQ biosynthesis mutants were complemented by pEL41 (Fig. 1), which has a 24-kb insert containing two *Hin*dIII fragments (38, 39). This insert was known to complement the 10 previously described *M. extorquens* AM1 MoxC mutants and the MoxD mutant UV46 (Fig. 1) (38, 39). These mutants were further divided into three groups on the basis of their complementation by p1130, containing a *Pst*I fragment that spans the internal *Hin*dIII site of pEL41, p1130C, containing the 3.4-kb *Pst*I-*Hin*dIII fragment of p1130, and p1130D, containing the 6.8-kb *Hin*dIII-*Pst*I fragment of p1130 (Fig. 1). In one group, the mutants were complemented by p1130 and p1130C, in another they were complemented by p1130 and p1130D, and in the third they were complemented by only p1130.

Further complementations with subclones and transposon insertions confirmed these three groups, MoxC, MoxT, and MoxO, and provided general locations for the genes defective in these mutants (Fig. 1). Thirty-five of the mutants, including the ten previously described MoxC mutants, were complemented by p1130C. These mutants were also not complemented by two subclones that divide this region, p1130C-PBg, containing a 1.8-kb *Pst*I-*Bgl*II fragment of p1130C, and p1130C-BgH, containing the adjoining 1.6-kb *Bgl*II-*Hin*dIII fragment (Fig. 1). The region of complementation was defined by two transposon insertions. All 35 mutants were complemented by p1130C containing a Tn*5lac* insertion 1.9 kb from the internal *Hin*dIII site in pEL41 (p1130C::Tn*5*-6; Fig. 1) but

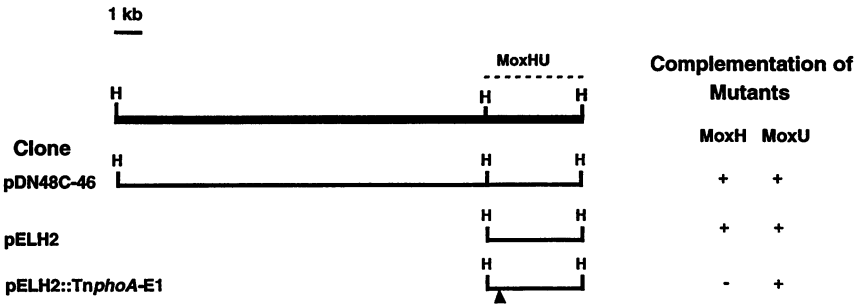


FIG. 2. Restriction map and complementation analysis of MoxH and MoxU mutants of *M. extorquens* AM1, using pDN48C-46 and its derivatives. The dotted line represents the maximum area for both genes; the triangle denotes Tn*phoA* insertion. H, *Hin*dIII.

not by p1130C containing a Tn*5lac* insertion 0.8 kb from the *Hin*dIII site (p1130C::Tn*5*-82). Therefore, the gene defective in these mutants should be located in the 1.9-kb region between Tn*5*-6 and the internal *Hin*dIII site. These mutants were all assigned to the MoxC complementation group.

Six of the mutants, including the MoxD mutant UV46, were complemented by p1130D, and their complementation group is proposed to be called MoxO. These mutants were also complemented by p1130D-HBg, which contains a 1.7-kb *Hin*dIII-*Bgl*II fragment of p1130D (Fig. 1). This group was further defined by two Tn*5lac* insertions in p1130D (p1130D::Tn*5*-136 and p1130D::Tn*5*-19) that abolished complementation of the MoxO mutants (Fig. 1).

Twenty-one mutants were complemented by pEL41 and p1130 but were not complemented by either p1130C or p1130D. This mutant class is proposed to be designated MoxT. These data suggest that the gene defective in the MoxT mutants spans the internal *Hin*dIII site of pEL41 and that the order of these complementation groups is MoxC-MoxT-MoxO (Fig. 1).

**H and U complementation groups.** Twenty-two of the new PQQ biosynthesis mutants were complemented by pDN48C-46, which contains a 23-kb *Hin*dIII insert with two fragments of 4.2 and 19 kb (Fig. 2). This clone was also known to complement UV48, which was previously designated in the MoxH complementation group (38, 39). It was previously reported that neither the 19-kb nor the 4.2-kb fragment of pDN48C-46 complemented UV48 (38), but we have shown that a subclone containing the 4.2-kb *Hin*dIII fragment (pELH2) complemented all of the mutants in this group (Fig. 2). The original subclone containing this fragment has been lost, and so the reason for this discrepancy is unknown, but it is possible that a small deletion was present in the original subclone. pELH2, containing a Tn*phoA* insertion (pELH2::Tn*phoA*-E1) 0.4 kb from the internal *Hin*dIII site of pDN48C-46, complemented only 2 of the 22 new mutants and did not complement UV48, dividing this region into two complementation groups. We propose to designate those mutants not complemented by pELH2::Tn*phoA*-E1 MoxH, since they include the previously described MoxH mutant UV48, and the mutants that are complemented by this plasmid are designated MoxU.

**Physiological characterization of PQQ biosynthesis mutants.** Representative mutants of the five complementation groups described above were grown on methylamine plus methanol (inducing conditions) and assayed for MDH activity and for the presence of PQQ in growth culture supernatants and cell extracts. In addition, extracts of the mutants were screened for cross-reaction with antibody against the alpha and beta subunits of MDH and the cytochrome $c_L$. All of the mutants showed identical phenotypes (Table 2). None of them showed detectable MDH activity or PQQ production in the culture supernatant or in cell extracts. In addition, all produced detectable but low levels of MDH subunits and wild-type levels of cytochrome $c_L$. These results confirm that these are PQQ biosynthesis mutants.

**Cross-complementation with *M. organophilum* DSM 760 and *M. organophilum* XX.** To compare the complementation groups identified for *M. extorquens* AM1 with those previously identified for *M. organophilum* DSM 760 and with the two *M. organophilum* XX mutants PT34 and PT47, cross-complementation experiments were carried out with *M. extorquens* AM1 clones and PQQ biosynthesis mutants of *M. organophilum* DSM 760 and *M. organophilum* XX (Table 3) and with *M. organophilum* DSM 760 clones and PQQ biosynthesis mutants of *M. extorquens* AM1 and *M. organophilum* XX (Fig. 3). pEL41, which complements the MoxC, MoxT, and MoxO mutants, complemented the *M. organophilum* XX mutants and four of the *M. organophilum* DSM 760 mutants, representing complementation groups PqqA, PqqB, PqqC, and PqqD. pDN48C-46, which complements the MoxH and MoxU groups, complemented the other two *M. organophilum* DSM 760 mutants, representing complementation groups PqqE and PqqF. Other clones were used to obtain specific complementation group correlations (Tables 3 and 4).

A new complementation group, MoxP, that seems to be equivalent to the PqqA group of *M. organophilum* DSM 760 but distinct from the MoxC group of *M. extorquens* AM1 was identified in *M. organophilum* XX. The *M. organophilum* XX mutants PT34 and PT47 were complemented by pEL41 and p1130C but not by p1130C::Tn*5*-6 or p1130C::Tn*5*-82, suggesting that they are in a separate complementation group from

TABLE 2. Characteristics of *M. extorquens* AM1 PQQ mutants[a]

| Strain | Complementation class | MDH activity (μmol/min/mg of protein) | PQQ[b] (μmol/mg of protein) | MDH subunit[c] | |
|---|---|---|---|---|---|
| | | | | Alpha | Beta |
| Wild type | | 0.33 | 0.1 | +++ | +++ |
| Mutants | | | | | |
| UV45 | MoxC | –[d] | – | + | + |
| EMS12 | MoxT | – | – | + | + |
| UV46 | MoxO | – | – | + | + |
| UV48 | MoxH | – | – | + | + |
| EMS7.42 | MoxU | – | – | + | + |

[a] All mutants had wild-type levels of cytochrome $c_L$.
[b] In crude extracts and in culture supernatant. –, not detectable (<0.02 nmol/mg of protein or nmol/ml of supernatant).
[c] By immunoblot analysis. +++, wild-type levels; +, low but detectable levels.
[d] –, not detectable.

TABLE 3. Complementation analysis of PQQ-defective mutants of *M. organophilum* DSM 760 and *M. organophilum* XX with cloned *mox* genes from *M. extorquens* AM1

| Plasmid | Class | Growth of transconjugants on methanol | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *M. organophilum* DSM 760 | | | | | | *M. organophilum* XX | |
| | | MTM1 (PqqA) | 452 (PqqB) | 474 (PqqC) | 71 (PqqD) | 430 (PqqE) | 530 (PqqF) | PT34 (MoxP) | PT47 (MoxP) |
| pEL41 | MoxPCTO | + | + | + | + | − | − | + | + |
| p1130C | MoxPC | + | − | − | − | − | − | + | + |
| p1130C::Tn*5*-6 | MoxC | − | | | − | | | − | − |
| p1130C::Tn*5*-82 | None | − | | | − | | | − | − |
| p1130D | MoxO | − | − | − | + | − | − | − | − |
| p1130D-HBg | MoxO | − | − | − | + | − | − | − | − |
| p1130D::Tn*5*-136 | None | | | | − | | | | |
| p1130D::Tn*5*-19 | None | | | | − | | | | |
| pDN48C-46 | MoxHU | − | − | − | − | + | + | − | − |
| pELH2 | MoxHU | − | − | − | − | + | + | − | − |
| pELH2::Tn*phoA*-E1 | MoxU | − | − | − | − | − | + | − | − |

the *M. extorquens* AM1 MoxC mutants, which are complemented by p1130C::Tn*5*-6. The MoxP group was originally designated VID (4) and later designated MoxP (25). MTM1, the PqqA mutant of *M. organophilum* DSM 760, showed the same complementation pattern with *M. extorquens* AM1 clones as did the *M. organophilum* XX mutants, suggesting that PqqA is equivalent to MoxP. In addition, the *M. organophilum* XX MoxP mutant PT34 was complemented by the *M. organophilum* DSM 760 clones pMO550, pMO500, and pMO552 but not by pMO551, which is the same complementation pattern observed for the *M. organophilum* DSM 760 PqqA mutants (Fig. 3). This finding provides further evidence that these two complementation groups are similar. None of the *M. organophilum* DSM 760 mutants tested were complemented by both p1130C and p1130C::Tn*5*-6, suggesting that no mutants equivalent to the MoxC group were identified in that strain. However, the *M. extorquens* AM1 MoxC mutant UV45 showed the same complementation pattern with the *M. organophilum* DSM 760 clones as did the MoxP and PqqA mutants, which indicates that these groups may all be similar. An analysis of the sizes of complementing DNA regions suggest that all of these groups are defective in the same gene (see Discussion and Table 4), and the gene defective in these mutants is proposed to be designated *pqqA*.

The *M. organophilum* DSM 760 PqqC mutant (mutant 474) appears to correspond to the MoxT complementation group, while the PqqB mutant (mutant 452) does not seem to correspond to any of the Mox groups (Table 4). The data supporting these conclusions are as follows. Both *M. organophilum* DSM 760 mutants were complemented by pEL41 but not by any other *M. extorquens* AM1 clones tested (Table 3). This is the same complementation pattern as for the *M. extorquens* AM1 MoxT group, suggesting that this *M. extorquens* AM1 DNA region contains at least two *pqq* genes, one of which is not represented by any of the *M. extorquens* AM1 PQQ biosynthesis mutants isolated. The MoxT mutant EMS12 was complemented by the same *M. organophilum* DSM 760 clones that complemented the PqqC mutant, but none of the *M. extorquens* AM1 PQQ biosynthesis mutants showed the same complementation pattern as the PqqB mutants (Fig. 3). The *M. extorquens* AM1 complementation group equivalent to PqqB of *M. organophilum* DSM 760 has been designated

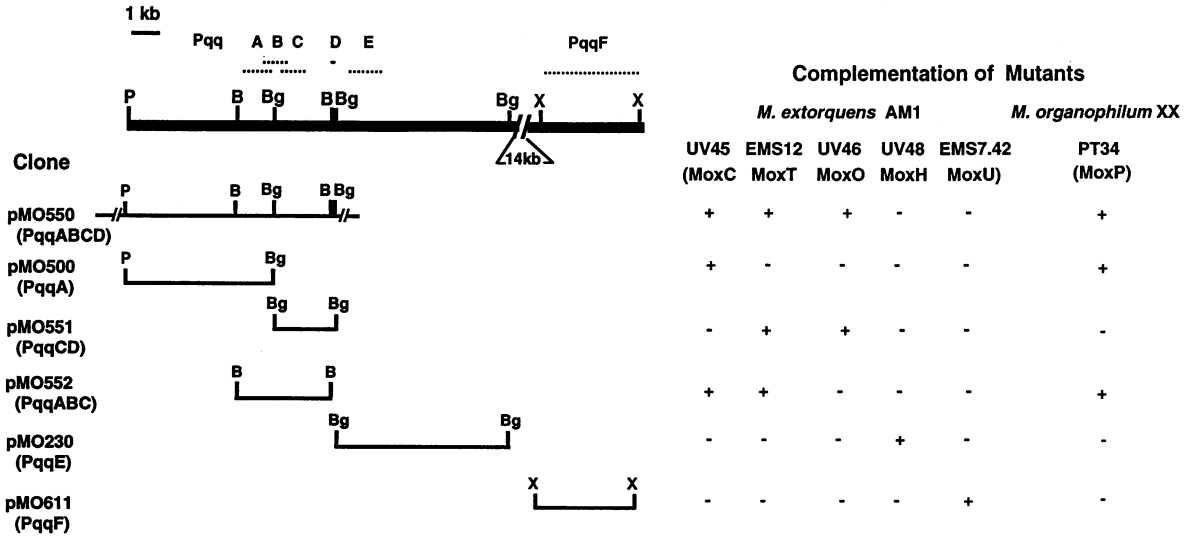


FIG. 3. Complementation analysis of PQQ biosynthesis mutants of *M. extorquens* AM1 and *M. organophilum* XX with cloned *pqq* genes from *M. organophilum* DSM 760 (map from reference 6). Dotted lines represent maximum gene sizes. X, *Xho*I; other abbreviations as in Fig. 1.

TABLE 4. Correlation of Pqq complementation groups in different methylotrophs

| *M. organophilum* XX | *M. organophilum* DSM 760[a] | *M. extorquens* AM1[b] |
|---|---|---|
| VID[c] | PqqD | MoxO |
| | PqqC | MoxT |
| | PqqB | MoxV |
| VID,[c] MoxP[b] | PqqA | MoxC[d] |
| | PqqE | MoxH |
| | PqqF | MoxU |

[a] Reference 6.
[b] References 25 and 28.
[c] Reference 4.
[d] See text.

MoxV (25) (Table 4). It is proposed that the gene defective in the MoxV mutants be designated *pqqB* and that the gene defective in the MoxT mutants be designated *pqqC*.

The *M. organophilum* DSM 760 PqqD group seems to be equivalent to the MoxO group (Table 4). PqqD mutant 71 was complemented by pEL41, p1130D, and p1130D-HBg, the same clones that complemented the MoxO mutants (Table 3). In addition, the PqqD mutant was not complemented by p1130D::Tn*5*-136 and p1130D::Tn*5*-19, which contain insertions that block complementation of the MoxO mutants, further suggesting that PqqD is equivalent to MoxO. This finding is supported by the fact that the complementation pattern of the *M. extorquens* AM1 MoxO mutant UV46 by the *M. organophilum* DSM 760 clones is the same as for the PqqD mutant; that is, MoxO mutants are complemented by pMO550 and pMO551 but not by pMO552 (Fig. 3). However, sequencing data show that this region contains two genes (see below).

The *M. organophilum* DSM 760 PqqE group seems to be equivalent to the MoxH group (Table 4). PqqE mutant 430 was complemented by pDN48C-46 and pELH2, the same clones that complemented the MoxH mutant UV48 (Table 3). The PqqE mutant was not complemented by pELH2::Tn*phoA*-E1, the clone containing a Tn*phoA* insertion that blocks complementation of the MoxH mutants, suggesting that PqqE is equivalent to MoxH. This conclusion is supported by the complementation of the MoxH mutant by the *M. organophilum* DSM 760 clone pMO230 but not pMO611, the same as for the PqqE mutant (Fig. 3). We propose to designate the gene defective in these mutants *pqqE*.

The *M. organophilum* DSM 760 PqqF group seems to be equivalent to MoxU (Table 4). The PqqF mutant 530 was complemented by pDN48C-46, pELH2, and pELH2:: Tn*phoA*-E1, the same clones that complemented the MoxU mutants (Table 3). The *M. extorquens* AM1 MoxU mutant EMS7.42 was complemented by the *M. organophilum* DSM 760 clone pMO611 but not pMO230, supporting the conclusion that PqqF is equivalent to MoxU (Fig. 3). We propose to designate the gene defective in these mutants *pqqF*.

**Nucleotide sequence of *pqqDGC'*.** The nucleotide sequence of the large *Bgl*II-*Hin*dIII fragment that was known to complement *M. extorquens* AM1 mutants in the MoxO complementation group was determined on both strands, using a series of plasmids including pCM187, pCM188, pCM198, and various subclones of these plasmids. This fragment was found to be 2,049 bp and contained two complete open reading frames and the N terminus of a third (Fig. 4 and 5). The first open reading frame, which traverses the internal *Bgl*II site, encodes a peptide of 29 amino acids. Genes that encode

```
AGATCTTCGACCGGCTCTGGGCGAGCCAGGGCGGCCTCTCGCTGAACGTGGTCGATGTCT     60
ATGTCGGCTACCTGCGCTCGAAGCTCTCGGATTTCGTGCGGCTGGGCGGCCCGGTAATCG    120
TCACGGTGCGCGGCAAGGGCTTCATGCTCGATCTGCGCGGCCAGGATTTCCGTCACTGAC    180
CCCCGCGGCATTTCGCTCCGAGCGCCGTATCCGACCGGATGGCATCAGGCCGGCGTCTCC    240
CGGTCTTTGCTTGAACGCGCTTTCTTCCGCCGAACCGGCGTCCACCTCGTCGGAAAGCGC    300
TCTCGGACCAAAGTTTAACCCGCGTCTCACGCGAAGTTATGCCGATCTCAGCGCGAACTT    360
GGGAAGAAACGGCAAGCTGGCTCTTGCAGGCGGGGCCGGAATTGGCGATATACCTCCGGC    420
GTGCGAAAACGAGTGCGTCGCCAGTATCTTACGATCCTCTTAATCAGTGGGATCGCGCGG    480
TTCCGGCGTTCCATAAAGTCGAGGAGAGACACCATGAAGTGGGCTGCCCCCATCGTTTCC    540
                                pqqD   M  K  W  A  A  P  I  V  S
GAGATCTGCGTCGGCATGGAAGTCACGAGCTACGAGTCGGCCGAGATCGACACCTTCAAC    600
 E  I  C  V  G  M  E  V  T  S  Y  E  S  A  E  I  D  T  F  N
TAAGGTGATTTGAGCCGGGTTGGGGTTGCAGGCATCAGCGGGTTTTCACCATGCATGTCG    660
                                      pqqG   M  H  V
                                  Tn5-136
                                     \/
TAATCCTGGGCTCGGCTGCGGGCGGCGGCGTTCCTCAATGGAACTGCCGCTGCTCCATCT    720
 V  I  L  G  S  A  A  G  G  G  V  P  Q  W  N  C  R  C  S  I
GCTCCCTGGCCTGGGCGGGCGATTCCCGCGTCAGGCCGCGCACGCAGTCGAGCATCGCAG    780
 C  S  L  A  W  A  G  D  S  R  V  R  P  R  T  Q  S  S  I  A
TCTCTCCTGACGGGGAACGCTGGCTCCTGCTGAACGCCTCTCCCGATATCCGTCAGCAGA    840
 V  S  P  D  G  E  R  W  L  L  L  N  A  S  P  D  I  R  Q  Q
TCCAGGCCAATCCGCAGATGCATCCGCGCGAGGGCCTGCGCCACTCGCCGATCCACGCGG    900
 I  Q  A  N  P  Q  M  H  P  R  E  G  L  R  H  S  P  I  H  A
TGCTGCTGACGAACGGCGACGTCGATCACGTTGCGGGCCTGCTGACCCTGCGCGAGGGCC    960
 V  L  L  T  N  G  D  V  D  H  V  A  G  L  L  T  L  R  E  G
AGCCCTTCACGCTCTACGCGACACCCGGCATCCTGGCCTCCGTCTCCGACAACCGCGTCT   1020
 Q  P  F  T  L  Y  A  T  P  G  I  L  A  S  V  S  D  N  R  V
TCGACGTGATGGCCGCCGACGTGGTGAAGCGGCAGACGATCGCCCTCAACGAGACCTTCG   1080
 F  D  V  M  A  A  D  V  V  K  R  Q  T  I  A  L  N  E  T  F
AGCCGGTGCCCGGCCTCTCGGTGACGCTGTTCTCCGTCCCCGGCAAGGTGCCGCTCTGGC   1140
 E  P  V  P  G  L  S  V  T  L  F  S  V  P  G  K  V  P  L  W
TGGAAGACGCCTCGATGGAGATCGGGGCGGAGACCGAAACCACGGTCGGCACGATGATCG   1200
 L  E  D  A  S  M  E  I  G  A  E  T  E  T  T  V  G  T  M  I
AGGCCGGGGGCAAGCGCCTCGCCTACATCCCCGGCTGCGCCCGGGTGACGGAGGATCTCA   1260
 E  A  G  G  K  R  L  A  Y  I  P  G  C  A  R  V  T  E  D  L
                                  Tn5-19
                                     \/
AAGCCCGCATCGCCGGCGCAGACGCGCTCCTGTTCGACGGCACGGTGCTGGAGGACGACG   1320
 K  A  R  I  A  G  A  D  A  L  L  F  D  G  T  V  L  E  D  D
ACATGATCCGCGCCGGTGTCGGCACCAAGACCGGCTGGCGCATGGGCCATATCCAGATGA   1380
 D  M  I  R  A  G  V  G  T  K  T  G  W  R  M  G  H  I  Q  M
ACGGCGAGACCGGCTCGATCGCGTCTCTCGCCGATATCGAGATCGGCCGACGGGTCTTCG   1440
 N  G  E  T  G  S  I  A  S  L  A  D  I  E  I  G  R  R  V  F
TTCACATCAACAACACCAATCCGGTCCTGATCGAGGATTCGTACGAGCGCGCGAGCGTCG   1500
 V  H  I  N  N  T  N  P  V  L  I  E  D  S  Y  E  R  A  S  V
AGGCGCGCGGCTGGACCGTCGCCCATGACGGCCTGACCCTCGATCTCTGATCAGGCTGAT   1560
 E  A  R  G  W  T  V  A  H  D  G  L  T  L  D  L  ---
GTCTTGGGAAGAGCCCGGTCTGGAAATTTAGTGCCGGACTGAATATGTTTTGCACGATCC   1620
AATCGTGCGGCAGCGGCCCTGCCCCGATCGGTACCGGGCCCCATTTAAAAATAAATCCAG   1680
GAAACGCGACTCGAAGCTCGGGGGAAACCGAACGCCATGACCGCCCAATTCCCGCCGCCC   1740
                                pqqC   M  T  A  Q  F  P  P  P
GTCCCGGACACCGAGCAACGCCTGCTGAGCCACGAGGAGCTTGAGGCGGCGCTCCGCGAT   1800
 V  P  D  T  E  Q  R  L  L  S  H  E  E  L  E  A  A  L  R  D
ATCGGTGCACGGCGCTACCACAACCTCCACCCGTTCCACCGGCTGCTGCACGACGGCAAG   1860
 I  G  A  R  R  Y  H  N  L  H  P  F  H  R  L  L  H  D  G  K
CTGTCGAAGGATCAGGTCCGGGCCTGGGCGCTCAACCGCTACTATTATCAGGCGATGATT   1920
 L  S  K  D  Q  V  R  A  W  A  L  N  R  Y  Y  Y  Q  A  M  I
CCGGTGAAGGATGCAGCGCTGCTGGCTCGCCTGCCGGATGCGCAGCTTCGCCGAATCTGG   1980
 P  V  K  D  A  A  L  L  A  R  L  P  D  A  Q  L  R  R  I  W
CGCCAGCGCATCGTCGATCACGACGGCGACCATGAGGGCGACGGCGGCATCGAGCGTTGG   2040
 R  Q  R  I  V  D  H  D  G  D  H  E  G  D  G  G  I  E  R  W
CTCAAGCTT                                                      2049
 L  K  L
```

FIG. 4. Nucleotide sequence of *M. extorquens* AM1 *pqqDGC'*. The relevant deduced amino acid residues are indicated below the nucleotide sequence. Termination codons (---) and sites of insertion for Tn*5*-136 and Tn*5*-10 (\/) are indicated.

similar peptides have been reported from *A. calcoaceticus* (*pqqIV*, 24 amino acids) and *K. pneumoniae* (*pqqA*, 23 amino acids) (14, 34). Seven amino acids, including the two amino acids thought to be important in providing the structural building blocks for PQQ, glutamate (E-16) and tyrosine (Y-20), are conserved in all three peptides (Fig. 6). These residues are separated by three amino acids in each case. The *M. organophilum* DSM 760 gene that has been suggested to be equivalent to *pqqIV* of *A. calcoaceticus* has been called *pqqD*

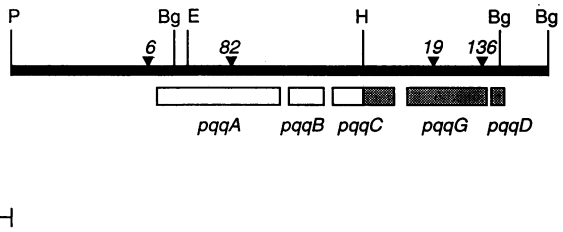


FIG. 5. *pqqDGCBA* region of *M. extorquens* AM1. Shaded boxes, sequenced regions; open boxes, assumed gene sizes in comparison with those from *K. pneumoniae* (34) and *A. calcoaceticus* (14). The sites of insertion of Tn*5lac* insertions (▼) are indicated (see Fig. 1). Transcription for *pqqD*, *pqqG*, and *pqqC* is right to left as shown. P, *Pst*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII.

(6), and we propose to designate this gene *pqqD* in *M. extorquens* AM1. The second open reading frame lies 47 bp downstream of *pqqD* and encodes a polypeptide of 299 amino acids. It shows substantial amino acid sequence identity to the open reading frames encoded by the second gene in the *pqq* clusters of *A. calcoaceticus* (*pqqV*, 36%) and *K. pneumoniae* (*pqqB*, 36%). This open reading frame has been designated *pqqG*, since no equivalent gene has been reported for *M. organophilum* DSM 760. The third open reading frame follows *pqqG* at a distance of 167 bp. Only the first 111 amino acids are known, since the open reading frame is truncated at the internal *Hin*dIII site. The 111 amino acids determined show 50 and 43% identity with the third gene in the *pqq* clusters of *A. calcoaceticus* (*pqqI*) and *K. pneumoniae* (*pqqC*), respectively. The gene encoding this open reading frame corresponds to the *M. extorquens* AM1 MoxT complementation group and is *pqqC*. The direction of transcription is from *pqqD* through *pqqG* and *pqqC*.

**Tn*5lac* insertions.** The sites of insertion of the two Tn*5lac* insertions that abolished complementation of the MoxO mutants were determined by sequencing. Both were found to lie within *pqqG*, with Tn*5*-136 being between nucleotides 701 and 702 and Tn*5*-19 being between nucleotides 1295 and 1296 (Fig. 4).

## DISCUSSION

The results presented here show that several *pqq* genes of *M. extorquens* AM1 are equivalent to and are clustered in the same gene order as *pqq* genes in other bacteria (Fig. 7). Complementation analysis between Pqq mutants of three methylotrophs (*M. extorquens* AM1, *M. organophilum* DSM 760, and *M. organophilum* XX) and DNA fragments of *M. extorquens* AM1 and *M. organophilum* DSM 760 identified seven possible complementation groups. However, our evidence suggests that two of these (MoxP and MoxC groups) represent mutants defective in the same gene. The *M. organophilum* XX mutants (MoxP group) appear to be equivalent to PqqA mutants, but the gene defective in the MoxC mutants is more difficult to assign. Although the complementation pattern with *M. organophilum* DSM 760 clones was the same for all three groups of mutants, one transposon insertion in an *M. extorquens* AM1 clone (p1130C::Tn*5*-6) complemented the MoxC mutants but did not complement the PqqA and MoxP mutants. However, the gene defective in the *M. extorquens* AM1 MoxC mutants has a minimum size of 0.7 kb (Fig. 1). The gene defective in the *M. organophilum* DSM 760 PqqA mutants is known to encode a polypeptide of 43 kDa (6) and therefore should be approximately 1.3 kb. However, the *M. organophilum* DSM 760 region that complements all three groups of mutants (MoxC, MoxP, and PqqA) is only 1.5 kb. Therefore, unless the genes overlap substantially, the complementing region is too small to contain both genes if they are different. It is not clear why p1130C::Tn*5*-6 does not complement PqqA and MoxP mutants and does complement MoxC mutants if they are defective in equivalent genes. However, the lack of complementation occurred only with mutants in heterologous strains. No MoxC mutants were isolated in *M. organophilum* DSM 760 or *M. organophilum* XX, and likewise, no PqqA or MoxP mutants were isolated in *M. extorquens* AM1. Therefore, it is possible that this particular insertion (Tn*5*-6) creates a problem with expression or function in the heterologous strains. This question can be resolved definitively only by sequencing and expression studies, but comparative data for other *pqq* clusters also support the hypothesis that the PqqA/MoxP and MoxC complementation groups define a single *pqq* gene (see below and Fig. 7), and we propose to designate this gene *pqqA*.

In addition to the six complementation groups identified, sequencing identified two genes (*pqqDG*) within the DNA fragment complementing the MoxO (PqqD) group, suggesting that a total of seven *pqq* genes are present in *M. extorquens* AM1 in two clusters, *pqqDGCBA* and *pqqEF* (Fig. 7). The sequencing data also show that *M. extorquens* AM1 mutants of the MoxO complementation group are defective in *pqqG*. The two Tn*5lac* insertions that abolish MoxO complementation (Tn*5*-19 and Tn*5*-136) were shown by sequencing to be located within *pqqG*. In addition, the smallest subclone that complemented MoxO mutants (p1130D-HBg) contains the complete *pqqG* gene but a truncated *pqqD* gene. This finding shows that, as in *K. pneumoniae pqqB* but unlike the case for *A. calcoaceticus pqqV*, the *M. extorquens* AM1 *pqqG* product is essential for PQQ biosynthesis (14, 34).

The same seven genes appear to be present in *M. organophilum* DSM 760. Although *pqqG* had not been previously identified in *M. organophilum* DSM 760, it is present, since *M. organophilum* DSM 760 clones containing the *pqqDC* region complement the *M. extorquens* AM1 mutants defective in *pqqG*. This region in *M. organophilum* DSM 760 does contain

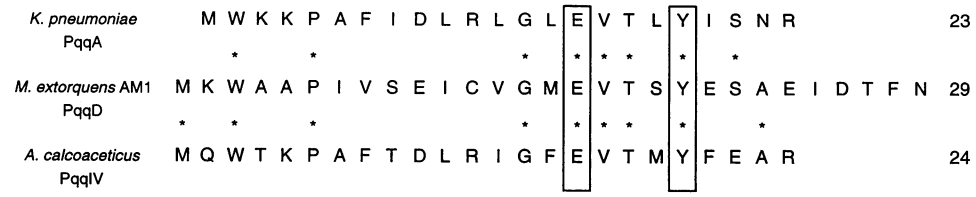


FIG. 6. Alignment of the peptides encoded by *K. pneumoniae pqqA*, *M. extorquens* AM1 *pqqD*, and *A. calcoaceticus pqqIV*. The peptides are aligned to maximize identity between them, indicated by *. Peptide length (amino acid residues) is indicated at the right. The conserved glutamate (E) and tyrosine (Y) residues are boxed.

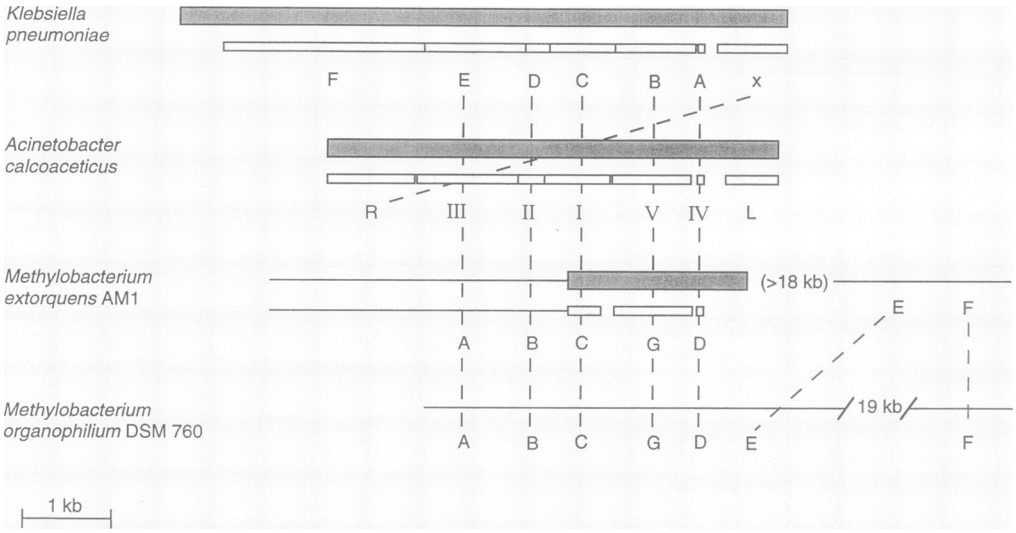


FIG. 7. Alignment of *pqq* gene clusters from *K. pneumoniae*, *A. calcoaceticus*, *M. extorquens* AM1, and *M. organophilum* DSM 760. Shaded boxes, regions that have been sequenced; open boxes, deduced open reading frames (gene designations are shown below); solid lines, DNA fragments known to encode the indicated genes through complementation analysis; vertical dashed lines, equivalent genes.

sufficient space between *pqqD* and *pqqC* (approximately 1 kb) to encode *pqqG* (Fig. 3). It may be that *pqqG* is nonessential in *M. organophilum* DSM 760. It is not clear at this time whether the *M. organophilum* DSM 760 PqqD mutant 71 is defective in *pqqD* or *pqqG*. Although the initial complementation data mapped this mutation to a 0.1-kb DNA region, suggesting that it was in *pqqD* (6), it showed the same complementation pattern with *M. extorquens* AM1 clones and Tn*5lac* insertions as the *M. extorquens* AM1 MoxO (*pqqG*) mutants. Further studies are necessary to resolve this discrepancy.

Although *pqqDGCBA* appear to be arranged similarly in the two *Methylobacterium* strains, *pqqE* and *pqqF* appear to be arranged differently (Fig. 7). In *M. organophilum* DSM 760, *pqqE* is located adjacent to *pqqD* and *pqqF* is approximately 19 kb away (6), while in *M. extorquens* AM1, the two genes are tightly clustered. Although it is not known where the *M. extorquens* AM1 DNA fragment that contains *pqqEF* is located with respect to the *pqqDGCBA* cluster, analysis of the known restriction fragments suggests they must be located at least 18 kb apart. At this time, sufficient data are not available to determine the order of *pqqE* and *pqqF* in *M. extorquens* AM1.

Less is known concerning the PQQ biosynthesis groups in *M. organophilum* XX. In this study, an *M. organophilum* XX MoxP complementation group has been identified and shown to be equivalent to the *M. organophilum* DSM 760 PqqA group. In addition, the same 2.1-kb *M. organophilum* XX DNA fragment that contains the gene equivalent to that defective in the PqqA mutants has been shown previously to be capable of complementing an *M. extorquens* AM1 MoxO mutant (4, 30). These data suggest that a gene functionally equivalent to *pqqG* is present in *M. organophilum* XX, linked to a gene equivalent to *pqqA*. It is not known whether the other five genes are also present in *M. organophilum* XX.

The sequence data presented here show that the first three genes of the *pqqDGCBA* cluster of *M. extorquens* AM1 are similar to the first three genes of the *pqq* clusters identified in *A. calcoaceticus* and *K. pneumoniae* (Fig. 7). Although only the first 111 amino acids of the *pqqC* open reading frame were determined, the equivalent genes in *A. calcoaceticus* and *K. pneumoniae* encode polypeptides of 252 and 251 amino acids, respectively. Therefore, approximately another 140 residues of the *M. extorquens* AM1 PqqC are expected to be encoded by the adjacent DNA fragment, which would place the 3′ end of *pqqC* approximately 0.4 kb beyond this *Hin*dIII site (Fig. 7). This allows a more precise mapping of *pqqA* and *pqqB*. If *pqqA* and *pqqB* of *M. extorquens* AM1 are equivalent in size and function to *pqqE* and *pqqD* of *K. pneumoniae* as has been suggested for *M. organophilum* DSM 760 (6), then they would fit well in the known region for the corresponding complementation groups (Fig. 7). Further sequencing will be required to determine whether *pqqA* and *pqqB* are equivalent to the corresponding genes in *A. calcoaceticus* and *K. pneumoniae*.

It is not known whether genes equivalent to *pqqE* and *pqqF* of the methylotrophs are present in *A. calcoaceticus* and *K. pneumoniae*. However, a fragment of *E. coli* DNA complements PqqE and PqqF mutants of *M. organophilum* DSM 760 (44), suggesting that functionally equivalent genes are present in *E. coli*. *E. coli* synthesizes an inactive glucose dehydrogenase apoprotein that can be activated with PQQ (18), and it is possible that some strains contain genes required for parts of the PQQ biosynthetic pathway. In at least one strain, mutants that are now able to produce PQQ can be isolated (7). Further work is under way to determine the functions of each of these genes involved in PQQ biosynthesis in *M. extorquens* AM1.

## ACKNOWLEDGMENTS

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