Sequence analysis of *pqq* genes required for biosynthesis of pyrroloquinoline quinone in *Methylobacterium extorquens* AM1 and the purification of a biosynthetic intermediate

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***Methylobacterium extorquens* AM1 produces pyrroloquinoline quinone (PQQ), the prosthetic group of methanol dehydrogenase. Two gene clusters have been shown to be required for PQQ biosynthesis in this micro-organism and complementation analysis has identified seven *pqq* genes, *pqqDGCBA* and *pqqEF*. The DNA sequence of *pqqDGC* was reported previously. This paper reports the sequence of the genomic region corresponding to *pqqC'BA*. For consistency, the nomenclature of *pqq* genes in *Klebsiella pneumoniae* will be followed. The new nomenclature for *pqq* genes of *M. extorquens* AM1 is *pqqABCDE* and *pqqFG*. In the genomic region sequenced in this study, two open reading frames were found. One of these encodes PqqE, which showed high identity to analogous *pqq* genes in other bacteria. PqqE also showed identity to MoaA and NifB in the N-terminal region, where a conserved CxxxCxYC sequence was identified. The sequence of the second open reading frame covered both the *pqqC* and *pqqD* regions, suggesting that both functions were encoded by this gene. It is proposed to designate this gene *pqqC/D*. The deduced amino acid sequence of the *pqqC/D* product showed identity to PqqC of *K. pneumoniae* and PqqI of *Acinetobacter calcoaceticus* in the N-terminal region, and to PqqD of *K. pneumoniae* and PqqII of *A. calcoaceticus* in the C-terminal region. A fragment of *M. extorquens* AM1 DNA containing only *pqqC/D* produced a protein of 42 kDa in *Escherichia coli*, which corresponds to the size of the deduced amino acid sequence of PqqC/D, confirming the absence of a separate *pqqD*. This genomic region complemented the growth of *pqqC* mutants of *M. extorquens* AM1 and *Methylobacterium organophilum* DSM 760 on methanol. As previously reported for *pqq* genes of *K. pneumoniae*, a *pqqC* mutant of *M. extorquens* AM1 produced an intermediate of PQQ biosynthesis, which was converted to PQQ by incubation with a crude extract from *E. coli* cells expressing PqqC/D. The intermediate was found in both crude extract and culture supernatant, and it was purified from the crude extract. The PqqC/D enzyme reaction appeared to require molecular oxygen and reduced nicotinamide adenine dinucleotides.**

Keywords: PQQ, pyrroloquinoline quinone, biosynthesis, methylotrophs

INTRODUCTION

Pyrroloquinoline quinone (PQQ) is the prosthetic group of several bacterial enzymes, including methanol de-

hydrogenase of methylotrophs and the glucose dehydrogenase of a number of bacteria (Anthony, 1993; Matsushita & Adachi, 1993). Genes involved in PQQ synthesis have been cloned from *Acinetobacter calcoaceticus*

Abbreviation: PQQ, pyrroloquinoline quinone.

The GenBank accession numbers for the sequences determined in this work are: L25889 for *pqqAB* and the 5' part of *pqqC/D*, and U72662 for the 3' part of *pqqC/D* and *pqqE*.

(Goosen *et al.*, 1989), *Klebsiella pneumoniae* (Meulenberg *et al.*, 1992), *Pseudomonas fluorescens* CHA0 (Schnider *et al.*, 1995), *Methylobacterium organophilum* DSM 760 (Biville *et al.*, 1989) and *Methylobacterium extorquens* AM1 (Morris *et al.*, 1994). In *A. calcoaceticus*, five genes were identified and sequenced, designated IV, V, I, II and III (Goosen *et al.*, 1989). In *K. pneumoniae*, genes analogous to those were identified and designated *pqqABCDE*, and in addition a sixth gene was found immediately downstream of *pqqE*, designated *pqqF* (Meulenberg *et al.*, 1992). In *Methylobacterium* strains, a five-gene cluster (designated *pqqDGCBA*) was identified by complementation analysis (Biville *et al.*, 1989; Morris *et al.*, 1994), and sequence data from *M. extorquens* AM1 showed that the first three of these genes (*pqqDGC*) were analogous to *pqqABC* of *K. pneumoniae* (Morris *et al.*, 1994). In *P. fluorescens*, genes analogous to *pqqFAB* of *K. pneumoniae* have also been sequenced (Schnider *et al.*, 1995). In all four sequences, a small gene is present that encodes a peptide of 22–29 amino acids, which contains conserved tyrosine and glutamate residues. Since tyrosine and glutamate are the probable precursors for PQQ synthesis (van Kleef & Duine, 1988; Houck *et al.*, 1991), it has been proposed that this peptide is the precursor from which PQQ is synthesized (Goosen *et al.*, 1992). However, the biochemical steps of PQQ synthesis are still unknown.

Velterop *et al.* (1995) examined PQQ synthesis *in vitro*. A series of experiments was carried out in which cell extracts of *Escherichia coli* containing all but one of the Pqq proteins were combined with those containing the missing Pqq protein. PQQ was produced in only one of these sets, that involving PqqC. *E. coli* cells containing a clone encoding all but the PqqC protein apparently produced an intermediate of PQQ, found both in the culture medium and in the cells. However, the amount of the intermediate was low and it was unstable.

In the work reported in this paper, the genomic region of *M. extorquens* AM1 proposed to contain *pqqC'BA* was sequenced. In order to develop a consistent *pqq* nomenclature, we followed the nomenclature of *pqq* genes in *K. pneumoniae* (Meulenberg *et al.*, 1992), which changes all of the *M. extorquens* AM1 *pqq* gene names except *pqqC* (see Table 2). In the sequenced region, we found only two open reading frames (ORFs), *pqqC/D* and *pqqE*. PqqC/D in *M. extorquens* AM1 appears to be a fusion of two Pqq proteins found in other bacteria, for instance, PqqC and PqqD in *K. pneumoniae*. A mutant of *M. extorquens* AM1 lacking PqqC/D produced an intermediate of PQQ as in the case of *K. pneumoniae*, but in much higher amounts. This intermediate was purified from the cell extract of the *pqqC* mutant of *M. extorquens* AM1.

METHODS

Bacterial strains and culture conditions. Strains and plasmids are listed in Table 1. *Methylobacterium extorquens* AM1rif and its mutant EMS12 were used as the wild-type and the

pqqC mutant strain, respectively (Morris *et al.*, 1994). Cells were grown as described previously, using 0.2% succinate, 0.2% methylamine hydrochloride plus 0.2% or 0.5% methanol (Morris *et al.*, 1994). *Escherichia coli* DH5 α harbouring pHT1KSac19f (see below) was cultured at 37 °C in Luria broth containing 50 μ g ampicillin ml⁻¹ to stationary phase.

DNA manipulations. Plasmid DNA from *E. coli* strains was prepared as described by Sambrook *et al.* (1989). Restriction enzyme digestion, DNA ligation and other DNA modifications were performed according to the vendors' recommendations.

DNA sequencing. A HindIII–PstI fragment was sequenced on both strands using the pUC universal and reverse primers and primers based on the determined sequences. Sequencing reactions and analyses were performed at the Sequencing Core Facility, California Institute of Technology, on an ABI 373A automated sequencer (Applied Biosystems). Oligonucleotides used for sequencing were synthesized at the Biopolymer Synthesis & Analysis Resource Center at California Institute of Technology. Homology analyses were carried out using the Genetics Computer Group (GCG) program.

Plasmid construction. A 1.3 kb *KpnI*–*SacI* fragment containing *pqqC/D* was blunted with T4 DNA polymerase and then ligated into the *HincII* site of pUC19. pHT1KSac19f, in which *pqqC/D* is under the control of the *lac* promoter, was identified by restriction mapping. From pHT1KSac19f, a 1.3 kb *BamHI*–*PstI* fragment was isolated and ligated with pRK310, a broad-host-range vector (Ditta *et al.*, 1985). pHT1KSac18 was a pUC18 derivative having the *KpnI*–*SacI* fragment in the opposite orientation to pHT1KSac19f (*pqqC/D* opposite to the *lac* promoter).

Preparation of crude extracts and culture supernatant. EMS12 was grown on minimal medium containing methylamine plus methanol to early stationary phase and *E. coli* DH5 α (pHT1KSac19f) was grown on LB medium containing ampicillin for more than 24 h. Cells and culture supernatant were separated by centrifugation at 4000 g for 5 min at 4 °C. Culture supernatant was filtered further with a 0.22 μ m filter when used in the assay for the intermediate. *M. extorquens* AM1 cells and *E. coli* DH5 α (pHT1KSac19f) cells were washed and suspended in 50 mM potassium phosphate buffer (pH 7.0) at a concentration of 1 g wet cells per 10 ml, and this preparation was passed through a French pressure cell twice at 20000 p.s.i. (138 MPa). This preparation was centrifuged at 12000 g for 10 min at 4 °C, and the supernatant was used as the crude extract.

Purification of the intermediate. One-tenth volume concentrated HCl was added to crude extract from EMS12 grown on minimal medium with methylamine and methanol, and the mixture allowed to stand on ice for 20 min. The resulting precipitate was removed by centrifugation at 12000 g for 10 min at 4 °C. The pH of the supernatant was adjusted to 4.5 with 10 M NaOH and passed through a Sep-Pak tC₁₈ vac, 10 g cartridge (Waters). The passed fraction was adjusted to pH 2 and passed through another Sep-Pak tC₁₈ vac cartridge. The intermediate adsorbed onto this cartridge and was eluted with 10% (v/v) methanol. The fraction was adjusted to pH 6.5 with 10 M NaOH and passed through a Sep-Pak QMA cartridge (Waters). This was then washed with 1 mM HCl, and the intermediate was eluted with 100 mM HCl. The eluted fraction was frozen at –20 °C, dried with a Speed Vac concentrator without heating, and then dissolved in 5 mM HCl. This fraction was analysed with a Hewlett Packard series II 1090 HPLC system equipped with a Hypersil BDS C-18 column (250 $\times$ 4 mm, Hewlett Packard). The eluent

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant trait(s)	Source or reference
<i>E. coli</i>		
DH5 α	r ⁻ m ⁺ <i>recA1 lacZYA</i> ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15	Bethesda Research Laboratories
S17-1	<i>recA thi pro</i>	Simon <i>et al.</i> (1983)
<i>M. extorquens</i>		
AM1rif		Nunn & Lidstrom (1986)
EMS12	<i>pqqC</i> mutant of AM1rif	Morris <i>et al.</i> (1994)
<i>M. organophilum</i> DSM 760		
452	<i>pqqB</i>	Biville <i>et al.</i> (1989)
474	<i>pqqC</i>	Biville <i>et al.</i> (1989)
Plasmids		
pRK310	Tc ^r IncP1	Ditta <i>et al.</i> (1985)
pUC18/19	Ap ^r <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pHT1KSac19f	pUC19 containing 1.3 kb <i>KpnI</i> – <i>SacI</i> fragment (<i>pqqC/D</i>)	This study
pHT1KSac18	pUC18 containing 1.3 kb <i>KpnI</i> – <i>SacI</i> fragment (<i>pqqC/D</i>)	This study
pHT1KSac	pRK310 containing 1.3 kb <i>Bam</i> HI– <i>Pst</i> I fragment from pHT1KSac19f (<i>pqqC/D</i>)	This study

used was 2% (v/v) acetonitrile in distilled water adjusted to pH 2.5 with phosphoric acid. The peak corresponding to the intermediate was determined by collecting fractions and assaying them as described below.

Detection and assay of the intermediate. The intermediate was detected after conversion to PQQ with crude extract of *E. coli* DH5 α (pHT1KSac19f). The reaction was performed in 100 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and 100 μ M NADPH at 30 °C. The crude extract was used in abundance (10 μ g or more protein of the crude extract per ml in the reaction mixture) to ensure that the intermediate was converted to PQQ completely. The reaction was stopped by dilution into the PQQ assay mixture (see below).

Assay of PQQ. PQQ content was determined with the apoenzyme of soluble glucose dehydrogenase purified from *E. coli* containing the cloned gene from *Acinetobacter calcoaceticus* (Cleton-Jansen *et al.*, 1989), which was a gift from N. Goosen. Samples containing PQQ were incubated with the apoenzyme (170 ng) in 250 μ l BisTris/HCl or PIPES/NaOH buffer (pH 6.5) containing 0.1% Triton X-100 and 4 mM calcium chloride for more than 5 min at room temperature. Then a 100 μ l portion was used to measure the activity spectrophotometrically as the reduction of phenazinemethosulfate and 2,6-dichlorophenol indophenol in 50 mM potassium phosphate (pH 6.5) as described previously (Matsushita *et al.*, 1995). The amount of PQQ was determined by comparison to a standard curve, generated with a PQQ solution whose concentration was checked by spectrophotometry using the molar absorption coefficient of 18400 M⁻¹cm⁻¹ at 249 nm (Duine *et al.*, 1980). Protein concentration was determined by a modified Lowry method (Dulley & Grieve, 1975).

Requirement for molecular oxygen. The reaction (100 μ l) was carried out at room temperature in a 2 ml vial open or closed with a rubber stopper. The head space of the closed vial was flushed with nitrogen gas for 5 min. The reaction was then started by addition of enzyme solution, which also had been

flushed with nitrogen gas. Aliquots (20 μ l) were taken by microsyringe, added to the PQQ assay mixture, and the PQQ produced was assayed as described above.

RESULTS AND DISCUSSION

Sequence analysis of *pqq* genes

A 3.4 kb *Hind*III–*Pst*I fragment of *M. extorquens* AM1 DNA known to complement *M. extorquens* AM1 and *M. organophilum* DSM 760 mutants in the PqqA complementation group (Morris *et al.*, 1994), was sequenced on both strands to complete the sequence for the *pqq* gene cluster (Fig. 1; Morris *et al.*, 1994). The complementation analysis had suggested that this genomic region should contain three *pqq* genes, *pqqC'BA* (Morris *et al.*, 1994). However, only two ORFs were observed in the regions responsible for complementation of *pqq* mutants: one in the *pqqCB* region and one in the *pqqA* region. Codon preference in these two ORFs was similar to that of the genes in *M. extorquens* AM1

Fig. 1. Restriction map of the 5.5 kb fragment containing the *pqqAB(C/D)E* region and the plasmids used in this study. Open arrows show ORFs of *pqq* genes. The filled triangle represents the Tn5-6 insertion (Morris *et al.*, 1994).

Table 2. Correlation of Pqq complementation groups in different micro-organisms

<i>K. pneumoniae</i>	<i>A. calcoaceticus</i>	<i>M. extorquens</i> AM1 (old)	<i>M. extorquens</i> AM1 (new)
<i>pqqA</i>	IV	<i>pqqD</i>	<i>pqqA</i>
<i>pqqB</i>	V	<i>pqqG</i>	<i>pqqB</i>
<i>pqqC</i>	I	<i>pqqC</i>	<i>pqqC/D</i>
<i>pqqD</i>	II	<i>pqqB</i>	<i>pqqC/D</i>
<i>pqqE</i>	III	<i>pqqA</i>	<i>pqqE</i>
<i>pqqF</i>	—	<i>pqqE</i>	<i>pqqF</i>
—	—	<i>pqqF</i>	<i>pqqG</i>

		10	20	30	40	50	60
PqqC/D	Mex	MTAQFPPVPDTEQRLLSHEELEAALRDIGARRYHNLHPFHRLLHDGKLSKDQVRAWALN					
PqqC	Kpn	MLITDTLSPQAFEEALRAKGA-FYHIHPYHIAMHNGDATRKQIQGVAN					
PqqI	Aca	MTQTPEALTTEQFKQAIIDKG-QYYHIYHPFVMMYEGKATQQQIQAVVAN					
			*	*	*	*	*
		70	80	90	100	110	120
		RYYYQAMIPVKDAALLARLPDAQLRRIWRQIRVDHGDGHEGDDGIERWLKLAEGVGFTRDYVLSTKGILS					
		RFYYQTTPKDAAIMANCPDAQTRRWQIRLDHGDGSHGEDGGIEAWLRLEAVGLSRDILLSEHVLPL					
		RYYYQINIPKDAAIMANCPDQVRQEWIQRMIDQGEYPDGGGREAWLRLEAVGLSREQVISEELVLP					
		*	*	*	*	*	*
		140	150	160	170	180	190
		ATRFSDVAYVHFVSERSLLEAIASSLTEMFSPTIISERVAGMLKNYDFITKDTLAYFDKRLTQAPRADDF					
		GVRFAVDAYLNFARRACWQEAACSSLTFLFAPQIHQSRLDSWPQHYPIWKEEGYFYFRSRLSQANRDVEH					
		GVRFAVDAYVNFRRASWREAASSSLTELFAPQIHQSRLDSWPQHYPIWIDDKGYEYFRSRLSQARRDVEH					
		*	*	*	*	*	*
		210	220	230	240	250	260
		ALDYVKRHATTPMEMQRAAIDALTFKCNVLWTQLDALYFAYVAPGMVPPDAWQPGEGGLVAETNSAEDSPA					
		GLALAKAYCDSAEKQNRMLEILQFKLDILWSMLDAMTAYALQRPYHTVTDKAAWHTTRLV					
		GLTITLDSFTTFEQQERMLEILQFKLDILWSILDALTLAYVHNEAPYHSVTSKRVRWHKGLFK					
		*	*	*	*	*	*
		280	290	300	310	320	330
PqqC/D	Mex	AASPAATTAEPFAFGSDVPRLPRGVRLRFDEVRNKHVLLAPERTFDLDDNAVAVLKLVD					
PqqD	Kpn	MQKTSIVAFRRGYRLQWEAAQESHVILYPEGMAKLNETAAILLVD					
PqqII	Aca	MNKEQFDLNLVPTWRQGYRFQFEPAQNGFVILYPEGMIKLNEAIGQYID					
			*	*	*	*	*
		340	350	360	370		
		GRNTVSQIAQILGQTYDADPAIIEADILFMLAGLAQKRVLER					
		GRRDVAAILIAMLNERF-PEAGGVDDVIEFLQIACQKWTTCREPE					
		GQNVSAIIAQLKQKFGDISDIDQVDVYMLVAKQHWIDLV					
		*	*	*	*		

Fig. 2. Comparison of the deduced amino acid sequence of PqqC/D of *M. extorquens* AM1 (Mex) to PqqC or PqqD of *K. pneumoniae* (Kpn), and PqqI or PqqII of *A. calcoaceticus* (Aca). Amino acids conserved among the three proteins are shown by asterisks under the sequences. Gaps inserted to maximize identity are shown by dashes.

available in the database (data not shown). These data suggested that *pqqC* and *pqqB* might be fused in *M. extorquens* AM1. To facilitate comparison among *pqq* genes of different micro-organisms, we propose to change the *pqq* nomenclature for *M. extorquens* AM1 to follow that of *K. pneumoniae* (Table 2). Therefore, the newly found ORFs are designated *pqqC/D* and *pqqE*.

The first ORF, *pqqC/D*, was predicted to be a 372-amino-acid polypeptide and its calculated molecular mass and isoelectric point were 41577 Da and 5.73, respectively. PqqC of *K. pneumoniae* and PqqI of *A. calcoaceticus* are composed of 251 and 252 amino acids, respectively, while PqqD of *K. pneumoniae* and PqqII of *A. calcoaceticus* are composed of 92 and 94 amino acids, respectively. Hence, the PqqC/D polypeptide is large enough to be a fusion of the two. Further supporting evidence for this idea came from sequence comparisons, which revealed that PqqC/D showed significant identity to PqqC of *K. pneumoniae* (44%) and PqqI of *A. calcoaceticus* (43%) in its N-terminus (residues 11–241,

Fig. 2). Likewise, the C-terminal portion (residues 285–366) showed significant identity to PqqD of *K. pneumoniae* (29%) and PqqII of *A. calcoaceticus* (30%). To determine whether the sequence was correct, and confirm that this region contained only one gene, SDS-PAGE analysis was carried out on a crude extract of *E. coli* DH5α cells containing pHT1KSac19f, the plasmid with a 1.3 kb *KpnI*–*SacI* insert containing only *pqqC/D* (Fig. 1). One significant, extra polypeptide band of around 42 kDa appeared in the crude extract of these cells but not in the controls (Fig. 3), suggesting that only one gene of the indicated size was present in this region. These results show that in *M. extorquens* AM1, *pqqC* and *pqqD* are fused into a single gene, which we propose to designate *pqqC/D*. This is consistent with the previous observation that no mutants of the PqqD complementation class were isolated from *M. extorquens* AM1 (Springer *et al.*, 1995).

PqqE was predicted to consist of 384 amino acids. Its calculated molecular mass and isoelectric point were 41714 Da and 6.42, respectively. It shows high identity

Fig. 3. SDS-PAGE of crude extract from *E. coli* containing different plasmids. Crude extract (20 µg protein) of *E. coli* harbouring pUC18, pHT1KSac18, pUC19 or pHT1KSac19f (lanes 1–4, respectively) was analysed by SDS-PAGE using a precast Tricine gel (10–20%, NOVEX). Proteins were stained by Coomassie Brilliant Blue R250. The arrowhead indicates the band of 42 kDa, presumably corresponding to PqqC. Lane M, Mark12 marker (NOVEX) containing 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6, 3.5 and 2.5 kDa proteins.

(45–46%) in residues 17–358 to *pqq* gene products from other bacteria (Fig. 4). In addition, by using the BLASTP program, identity was observed to MoaA and other related proteins, 39–56% and 16–47% in residues 18–35

and 55–92, respectively (see Table 3). Residues 28–45 and 109–135 of PqqE also showed identity to NifB from *Enterobacter agglomerans* and *K. pneumoniae* (see Table 3). MoaA is a protein involved in an early step in the biosynthesis of the molybdopterin cofactor (Rajagopalan & Johnson, 1992; Rivers *et al.*, 1993) and NifB is involved in an early step in the biosynthesis of the cofactor of nitrogenases, FeMo-, FeV- and FeFe-cofactors. NifB has recently been shown to incorporate iron and sulfur from its metabolic product, termed NifB-co, to the cofactors of dinitrogenases (Allen *et al.*, 1995), and it has been suggested that the role of this family of proteins is to donate metal atoms to the respective cofactor (Menendez *et al.*, 1995). All of these sequences listed in Table 3, including PqqE, contain a conserved CxxxCxYC sequence, which is proposed to anchor an iron–sulfur cluster or some type of metal-binding site (Menendez *et al.*, 1995). MoaA proteins have a second cysteine-rich region in their C-terminus, with the motif FCxxC(13x)C (Menendez *et al.*, 1995). The C-terminal region of PqqE is also cysteine-rich and almost all of these cysteine residues were conserved in all the corresponding Pqq proteins (Fig. 4), although the motif was different from that of MoaA. If PqqE is involved in synthesis of a cofactor, the identity of the enzyme containing that cofactor is unknown.

It has previously been shown that one transposon insertion (Tn5-6) in p1130C, a plasmid containing *pqq(C/D)E*, can differentiate *pqqE* of *M. extorquens* AM1 from the corresponding gene (previously called *pqqA*) of *M. organophilum* DSM 760. This insertion

		10	20	30	40	50	60
PqqE	Mex	MNAPTAPSPVDVIPAPVGLLAELTHRCPLRC	PCYCSNPLDRLRRSAELDTQTWLRVLTEA				
PqqIII	Aca	MTEGVGLPLWLLAELTYRCPLQCP	PCYCSNPLDYAQHKNELTQEWFDVFDQA				
PqqE	Kpn	MSQSKPTVNPPLWLLAELTYRCPLQCP	PCYCSNPLDFARQDKELTTEQWIEVFRQA				
Pqq	Ehe	MTDHAPTVPNPPLWLLAELTYRCPLQCP	PCYCSNPLDFAAQEKELSTEQWIEVFRQA				
			..*****.*****.*****.				
		70	80	90	100	110	120
		AGLVHVLHSGGEPTRAPDIVEITAKCAELGLYSLNLTSGVGGALAKLDALYDVLGHVQLSVQGVDAANA					
		RQMGAVQLGFSGGEPVLRQDLQLVAHAHQGFYTNLTSGMGLTEQRIADLKQAGLDHIQVSFQASDPVFN					
		RAMGSVQLGFSGGEPVLRQDLQLVAHAHQGFYTNLTSGMGLTEQRIADLKQAGLDHIQVSFQASDEVFN					
		RAMGSVQLGFSGGEPVLRQDLQLVAHAHQGFYTNLTSGMGLTEQRIADLKQAGLDHIQVSFQASDEVFN					
		..*.....*...*					
		140	150	160	170	180	190
		EKIGGLKNAQPKMQFAARVTEGLPLTLNSVIHRGNIHEVPGFIDLAVKLGAKRLEVAHTQYYGWAVNRA					
		DALAGSKHAFQKQYEMCRVLKKYDYPMVLFVHRHNDQIEQIEELCLNADTVELAICQFYGWAFNLRQ					
		AALAGNKKAFQQLAMAKAVKARDYPMVLFVHRHNDQIEQIEELCLNADTVELAICQFYGWAFNLRQ					
		AALAGSAKAFQQLTMAKAVKALGYPMVLFVHRHNDQIEQIEELCLNADTVELAICQFYGWAFNLRQ					
		...*...*					
		210	220	230	240	250	260
		ALMPDKSQVDESIRIVEAARERLKGQL---VIDLVVPDYAKYKACAGGWRKLMNVTPQGVLPCHAAET					
		GLLPTQEQQLTRAERITNEYREKLKAQNHPCKLIFVVPDYEEERPKACMNGWKIFFTVAPDGMALPCHAAEQ					
		GLLPTREQTARAQVQVADYRQKMAASGNLTNLLFVTPDYEEERPKGCMGGWSIFLSVTPEGTALPCHSARQ					
		GLLPTREQTAKAESVQRYRERMAVDGKLANLLFVTPDYEEERPKGCMGGWGAIFMSVTPEGMALPCHSARQ					
		..*...*					
		280	290	300	310	320	330
		IPGLEFWYVTDHALGEIWTSHGRFAAYRGTSWMKEPCRSRCDREKDWGGCQCQALALTGDAANTDPACLSLP					
		LP-ISFPNVREHKLSDIWKSTGFNFHFRGDWMPGCRSCPDKDRDFGGCQCQAYMLTGDAAANADPVCCKSP					
		LP-VAFPVLEQSLQESIWDSPGFNRYRGYDWMPEPCRSRSCDEKEDFGGCQCQAFMLTGSADNADPVCCKSP					
		LP-VKFPVLEHDLHIWVHSPGFNRYRGYDWMPEPCRSRSCDEKEDFGGCQCQAFMLTGSADNADPVCCKSP					
		..*...*					
		350	360	370	380		
		LHAKMRDLAKEEAETPPDYIYRSIGTINVQNPLSEKAPL					
		YHQMIEQARAESQLVAPLQ--NLVFRNRSNKSLSATQNPVHTITDI					
		HHHKILEARREACSDIKVS--QLQFRNRTRSQ--LIYQTRDL					
		HHGTIL--AAREANRTQLGIDQLFRNQANSR--LFYKG					
		..*...*					

Fig. 4. Comparison of the deduced amino acid sequence of PqqE of *M. extorquens* AM1 (Mex) to PqqE of *K. pneumoniae* (Kpn), PqqIII of *A. calcoaceticus* (Aca) and the product of a *pqq* gene of *Erwinia herbicola* (Ehe) (Liu *et al.*, 1992). Asterisks under the sequences show the amino acids conserved in all four proteins, and dots show the amino acids conserved in three proteins. Gaps are shown as dashes. The C--C-YC sequence conserved in MoaA and NifB proteins is in boldface.

Table 3. Alignment of PqqE from *M. extorquens* AM1 with the related *pqq* gene products, MoaA-related proteins and NifB

Homology analysis of PqqE was carried out using the BLASTP program in the GCG program. Only the region of interest is shown.

Protein	Organism*	Position	Conserved domain	Database ref.
PqqE	Mexto	20–40	LLAELTHRCPLRCFYCSNP	U60993
PqqE	Kpneu	14–34	LLAELTYRCPLQCFYCSNP	X58778
Pqq	Eherb	14–34	LLAELTYRCPLQCFYCSNP	M94448
PqqIII	Acalc	11–31	LLAELTYRCPLQCFYCSNP	X06452
Pqq?	Hinfl	40–51	KCNLDCPWCDTP	U32798
MoaA	Mther	14–29	LRISVTGRCNVSCIYC	X87968
Cnx2	Athal	77–92	LRISLTERCNLRCCYC	Z48047
Moa	Celeg	7–22	LRISLTEKCNFRCLYC	Z46267
MoaA	Ecoli	16–31	LRLSITDVCNFRCTYC	X70420
MoaA	Anico	47–58	LTDKCNLRCTYC	X78980
Aor4	Pfuri	4–19	LYLEITSRCNLRCEMC	X79777
MoaA	Hinfl	25–40	LRLSITDQCNFRCTYC	U32840
NarA	Bsubt	19–34	LRISVTDRCNFRCTYC	Z35277
NifB	Eagglo	28–40	CNLQCNVCNRKYD	Z49943
NifB	Kpneu	54–66	CNLQCNVCNRKFD	M15545
Consensus:			C---C-YC	

* Mexto, *Methylobacterium extorquens* AM1; Kpneu, *Klebsiella pneumoniae*; Eherb, *Erwinia herbicola*; Acalc, *Acinetobacter calcoaceticus*; Hinfl, *Haemophilus influenzae*; Mther, *Methanobacterium thermoautotrophicum*; Athal, *Arabidopsis thaliana*; Celeg, *Caenorhabditis elegans*; Ecoli, *Escherichia coli*; Anico, *Arthrobacter nicotinovorans*; Pfuri, *Pyrococcus furiosus*; Bsubt, *Bacillus subtilis*; Eagglo, *Enterobacter agglomerans*.

complements *pqqE* mutants of *M. extorquens* AM1 but not the *pqqA* mutant of *M. organophilum* DSM 760 (Morris *et al.*, 1994). We determined the inserted position by sequencing p1130C::Tn5-6, and found that it occurred inside the ORF of *pqqE*, that is 23–25 amino acids before the C-terminus (see Fig. 1), and that eight nucleotides were duplicated in both ends of the transposon. This C-terminal region is not conserved in these proteins (Fig. 4), and the site of the insertion suggests that this region is not necessary for PqqE function in *M. extorquens* AM1. However, this region is apparently important for function of the *M. extorquens* AM1 PqqE in *M. organophilum* DSM 760.

Complementation analysis of mutants of *M. organophilum* DSM 760

The 1.3 kb *KpnI*–*SacI* fragment containing *pqqC/D* (Fig. 1) complemented growth on methanol of *pqqC* mutants of *M. extorquens* AM1 (mutant EMS12), and *M. organophilum* DSM 760 (mutant 474). However, growth on methanol of the *pqqD* (previously *pqqB*) mutant of *M. organophilum* DSM 760 (mutant 452), was not complemented by the fragment although the subclone containing *pqqAB(C/D)E* of *M. extorquens* AM1 did complement these mutants (Morris *et al.*, 1994). The reason for this discrepancy is unknown, but the data suggest that in *M. organophilum* DSM 760,

functional expression of the PqqD portion of PqqC/D requires the presence of *pqqE*. No *pqqD* mutants have been identified in *M. extorquens* AM1 (Springer *et al.*, 1995).

Production of an intermediate by the *pqqC* mutant and activity to convert the intermediate to PQQ in crude extract of *E. coli* cells

Velterop *et al.* (1995) reported that *E. coli* cells containing a plasmid with all of the *pqq* genes except *pqqC* seemed to produce an intermediate of PQQ in both the culture medium and cells. This intermediate could be converted to biologically active PQQ by extracts containing only PqqC, suggesting that PqqC catalyses the last step in PQQ synthesis. We examined production of an intermediate by a *pqqC* mutant of *M. extorquens* AM1, EMS12 (Morris *et al.*, 1994). Detection of the intermediate was assessed after its conversion to PQQ using a crude extract of *E. coli* harbouring pHT1KSac19f, producing PqqC/D (Fig. 3). Specific activity in the *E. coli* crude extract was estimated to be 20 pmol min⁻¹ (mg protein)⁻¹ with 20 pmol of the partially purified intermediate in a 20 µl reaction mixture (see below). The activity was not observed in crude extract of *E. coli* containing the following controls: pUC18, pUC19 or pHT1KSac18, in which the orientation of *pqqC/D* to the *lac* promoter is opposite to pHT1KSac19f. The pH

Fig. 5. HPLC profile and absorption spectrum of the intermediate. Partially purified intermediate (the amount estimated was 100 pmol) was analysed with a Hypersil BDS C18 column (250 × 4 mm). The eluent used was 2% (v/v) acetonitrile in distilled water adjusted pH to 2.5 with phosphoric acid. Flow rate was 1.0 ml min⁻¹. mAU, milli-absorbance units.

optimum for this reaction was found to be around 8. The intermediate was detected in both the culture medium and crude extract of EMS12, and estimated to be 20–80 nM and 100–500 pmol (mg protein)⁻¹, respectively, in different batches, by using excess crude extract or incubating for longer periods. The total amount of the intermediate in crude extract was estimated to be slightly larger than that in the culture supernatant, 65–90 nmol (l culture)⁻¹, in contrast to the result in *K. pneumoniae*, where it was found mostly in the culture supernatant (Velterop *et al.*, 1995). The amounts of this intermediate are apparently much higher in *M. extorquens* AM1 than in *K. pneumoniae*, as might be expected since the amount of PQQ produced is much higher in *M. extorquens* AM1 (Ramamoorthi & Lidstrom, 1995) than in *K. pneumoniae* (Velterop *et al.*, 1995).

Purification of the intermediate

The intermediate was expected to be similar to PQQ, since one enzyme, PqqC/D, could convert it to PQQ, suggesting it might contain three carboxyl groups. However, attempts to purify the intermediate from culture supernatant using anion-exchange columns failed because recovery of the activity was very low (data not shown). However, the intermediate adsorbed on a reversed-phase material, Sep-Pak tC₁₈, at acidic pH near 2, but not above pH 3. These properties were used to design a purification scheme, starting with the crude extract of EMS12 (see Methods). Fig. 5 shows the chromatogram of HPLC analysis of the intermediate, and its absorption spectrum. The absorption maxima at pH 2.5 were 218, 274 and 520 nm. This compound was stable at acidic pH, but unstable at alkaline pH and to heat.

Effect of EDTA, NAD(P)H and molecular oxygen

The enzyme activity which converted the PQQ intermediate to PQQ was lowered after gel filtration with a Sephadex G-25 column or dialysis (data not shown),

Fig. 6. Requirement for oxygen in the PqqC/D reaction. The reaction (100 µl) was carried out in an open vial (●) or a closed vial containing a nitrogen atmosphere (○). At the indicated times, a 20 µl aliquot was taken by a microsyringe for assay (see text). The arrow indicates the time when the closed vial was opened to the air.

suggesting that the reaction required a low-molecular-mass compound(s). This decreased activity was restored or even enhanced by the addition of NADH or NADPH in the presence of EDTA. Without EDTA, NAD(P)H showed an inhibitory effect in high concentration (1 mM). The reaction was saturated for NADPH at 100 µM, and NADH at 500 µM.

An apparent requirement of molecular oxygen for the reaction was observed. If the reaction was carried out under a nitrogen atmosphere in a 2 ml vial closed with a rubber stopper, production of PQQ was substantially reduced, to about 20% of that in an open vial (Fig. 6). Normal production of PQQ was recovered after opening the vial to air (Fig. 6). Sodium cyanide and sodium azide (2 mM each) did not affect the activity.

These data imply that PqqC/D may be a mono-oxygenase or dioxygenase. However, the PqqC/D sequence does not show identity to any monooxygenases or dioxygenases in the database. Confirmation of the reaction will require determination of the structure of the intermediate, purification of PqqC/D, and investigation of its biochemical characteristics. These sets of experiments are now in progress.

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