***pqqA* is not required for biosynthesis of pyrroloquinoline quinone in *Methylobacterium extorquens* AM1**

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***Methylobacterium extorquens* AM1 is a facultative methylotroph that oxidizes methanol via the pyrroloquinoline quinone (PQQ)-linked enzyme methanol dehydrogenase. In *M. extorquens* AM1 and other PQQ-synthesizing bacteria, several genes are involved in the synthesis of PQQ and one of these, *pqqA*, has been proposed to encode a peptide precursor of PQQ. In other PQQ-synthesizing bacteria, *pqqA* is required for PQQ production. In this study, it is shown that both deletion and insertion mutants of *pqqA* in *M. extorquens* AM1 grow normally on methanol and produce PQQ. The level of PQQ production is reduced in the insertion mutant, but it is sufficient to allow normal growth on methanol. These results suggest either that a different peptide in *M. extorquens* AM1 can substitute for PqqA in *pqqA* mutants, or that PqqA-like peptides may not be obligatory precursors of PQQ. In addition, it is shown that the methanol oxidation transcriptional regulator gene, *mxhM*, is required for normal methanol induction of PQQ synthesis.**

Keywords: pyrroloquinoline quinone, PQQ, methylotrophs, *pqqA*

INTRODUCTION

Pyrroloquinoline quinone, PQQ, is the cofactor of methanol dehydrogenase (MDH), which is the enzyme that oxidizes methanol to formaldehyde in Gram-negative methylotrophic bacteria (Anthony *et al.*, 1994). Several enzymes besides MDH found in non-methylotrophic bacteria also contain PQQ (Matsushita & Adachi, 1993). The genes involved in biosynthesis of PQQ have been cloned and sequenced from *Acinetobacter calcoaceticus* (Goosen *et al.*, 1989), *Klebsiella pneumoniae* (Meulenberg *et al.*, 1992), *Pseudomonas fluorescens* (Schnider *et al.*, 1995) and *Methylobacterium extorquens* AM1 (Morris *et al.*, 1994; Springer *et al.*, 1996; Toyama *et al.*, 1997). In *K. pneumoniae*, the genes are termed *pqqABCDEF* (Meulenberg *et al.*, 1992). Tyrosine and glutamate have been shown to be the precursors of PQQ synthesis (Kleef & Duine, 1988; Houck *et al.*, 1991), but the pathway is still unknown.

An open reading frame encoding a short peptide (22–29 amino acids) is found at the start of the known *pqq* gene

clusters (Goosen *et al.*, 1989; Meulenberg *et al.*, 1992; Morris *et al.*, 1994; Schnider *et al.*, 1995; Gomelsky *et al.*, 1996). The gene encoding this peptide is required for PQQ synthesis in *A. calcoaceticus* (Goosen *et al.*, 1992), in *Escherichia coli* containing the *K. pneumoniae pqq* genes (Meulenberg *et al.*, 1992), and in *K. pneumoniae* (Velterop *et al.*, 1995). The deduced amino acid sequences of these peptides showed some similarity and included conserved tyrosine and glutamate residues (Goosen *et al.*, 1989; Meulenberg *et al.*, 1992; Morris *et al.*, 1994; Schnider *et al.*, 1995; Gomelsky *et al.*, 1996). In addition, another gene required for PQQ synthesis, *pqqF*, shows similarity to a family of proteases that might be involved in processing the peptide (Meulenberg *et al.*, 1992). Therefore, it has been proposed that the peptide might be the precursor of PQQ synthesis (Goosen *et al.*, 1992; Meulenberg *et al.*, 1992).

In *Methylobacterium extorquens* AM1, the genes for PQQ synthesis are found in two clusters, *pqqAB(C/D)E* and *pqqFG* (Toyama *et al.*, 1997). These gene designations standardize the nomenclature with that of *K. pneumoniae*. These genes in *Methylobacterium* strains were formerly called *pqqDGCBA* (Morris *et al.*, 1994) and *pqqEF* (Springer *et al.*, 1996), respectively. In *M. extorquens* AM1, *pqqC* and *pqqD* are not separate genes. Instead, they are fused into a single gene, *pqqC/D*

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

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 oriT</i> integrated RP4-2-Tc::Mu-Km::Tn7	Simon <i>et al.</i> (1983)
GM2163	F ⁻ <i>dam-13::Tn9</i> (Cam ^r) <i>dcm-6 mcrA</i>	New England Biolabs
<i>M. extorquens</i>		
AM1 rif	Wild-type	Nunn & Lidstrom (1986)
UV45	<i>pqqE</i>	Nunn & Lidstrom (1986)
EMS12	<i>pqqC</i>	Morris <i>et al.</i> (1994)
UV46	<i>pqqB</i>	Nunn & Lidstrom (1986)
3b	Δ [<i>mxhM pqqAB</i> (C/D)E]	This study
D2	<i>pqqA::Km</i>	This study
Plasmids		
pRK310	Tc ^r IncP1, broad-host-range vector	Ditta <i>et al.</i> (1985)
pUC18/19	Ap ^r <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pAYC61	Ap ^r Tc ^r <i>oriT</i> , suicide vector	Chistoserdov <i>et al.</i> (1994)
pALS519b/f	pUC19 containing 10 kb <i>Pst</i> I fragment [<i>mxhDM</i> and <i>pqqAB</i> (C/D)E]	This study
pALS5Kan61b/f	pALS519b/f::Km ^r (<i>Bgl</i> II site) Δ 4.1 kb <i>Bgl</i> II fragment (<i>mxhD</i> Δ [<i>mxhMpqqAB</i> (C/D)E])	This study
pHT1BH19	pUC19 containing 3.8 kb <i>Bam</i> HI– <i>Hind</i> III fragment (<i>mxhM</i> and <i>pqqAB</i>)	This study
pHT2SacH19	pUC19 containing 1.8 kb <i>Sac</i> II– <i>Hind</i> III fragment	This study
pHT2SacHKan61	pAYC61 containing the 1.8 kb <i>Sac</i> II– <i>Hind</i> III fragment of pHT1BH19 with Km ^r inserted into the <i>Bgl</i> II site in <i>pqqA</i>	This study
pHT3BH19	pUC19 containing 3.8 kb <i>Bam</i> HI– <i>Hind</i> III fragment deleted for <i>pqqA</i>	This study
pHT3SacH19	pUC19 containing 1.8 kb <i>Sac</i> II– <i>Hind</i> III fragment deleted for <i>pqqA</i>	This study
pHT1	pRK310 containing 7.4 kb <i>Bam</i> HI– <i>Pst</i> I fragment [<i>mxhM</i> and <i>pqqAB</i> (C/D)E]	This study
pHT2	pRK310 containing 5.4 kb <i>Bam</i> HI– <i>Pst</i> I fragment [<i>pqqAB</i> (C/D)E]	This study
pHT3	pRK310 containing 5.1 kb <i>Bam</i> HI– <i>Pst</i> I fragment (<i>pqq</i> (C/D)E)	This study
pHT3L	pRK310 containing 7.1 kb <i>Bam</i> HI– <i>Pst</i> I fragment [<i>mxhM</i> and <i>pqq</i> (C/D)E]	This study
pHT4	pRK310 containing 5.3 kb <i>Bam</i> HI– <i>Pst</i> I fragment [<i>pqqB</i> (C/D)E]	This study
pHT4L	pRK310 containing 7.3 kb <i>Bam</i> HI– <i>Pst</i> I fragment [<i>mxhM</i> and <i>pqqB</i> (C/D)E]	This study
pHT2Ns	pRK310 containing 0.5 kb <i>Bam</i> HI– <i>Pst</i> I fragment (<i>pqqA</i>)	This study
pHT2SacH	pRK310 containing 1.8 kb <i>Bam</i> HI– <i>Hind</i> III fragment (<i>pqqAB</i>)	This study
pHT4SacH	pRK310 containing 1.7 kb <i>Bam</i> HI– <i>Hind</i> III fragment (<i>pqqB</i>)	This study

(Toyama *et al.*, 1997). Transcriptional analysis of *pqqA* in *M. extorquens* AM1 showed that a short mRNA containing only *pqqA* was produced at a much higher level than the mRNA for *pqqAB* (Ramamoorthi &

Lidstrom, 1995). Similar results were obtained in the case of *K. pneumoniae* by using *pqq*–Tn5*lacZ* operon fusions on the chromosome (Velterop *et al.*, 1995). These results are also consistent with the hypothesis that

PqqA is a PQQ precursor, because stoichiometric amounts rather than catalytic amounts are required if the peptide is the substrate for PQQ synthesis.

As a first step to examine whether the peptide is the precursor and to elucidate the biochemical steps of PQQ biosynthesis, a deletion mutant of *M. extorquens* AM1 lacking *pqqAB(C/D)E* was constructed. This mutant was then complemented by a plasmid that contained all of the deleted genes except *pqqA*. Surprisingly, the transformed mutant showed wild-type growth on methanol and significant PQQ production, suggesting that in *M. extorquens* AM1, *pqqA* is not required for PQQ production.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. Genes contained in key plasmids are illustrated in Fig. 1.

Media and growth conditions. *M. extorquens* AM1 rif and its mutants were grown on the minimal medium described previously (Morris *et al.*, 1994), containing 0.5% (v/v) methanol, 0.2% (w/v) methylamine hydrochloride, or 0.46% (w/v) succinic acid disodium salt hexahydrate. When required, PQQ (Fluka) was added to 1 µM. *E. coli* was grown on LB medium. Antibiotics were used as following amounts (µg ml⁻¹): rifampicin, 20; kanamycin, 25; tetracycline, 10; ampicillin, 50. When production of PQQ was measured, *M. extorquens* AM1 cells were pre-cultured in 10 ml minimal medium with succinate and appropriate antibiotics, and cultured in

100 ml minimal medium containing appropriate carbon source without antibiotics.

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 vendors' recommendations. Oligonucleotides were synthesized at the Biopolymer Synthesis & Analysis Resource Center at California Institute of Technology. Sequencing reactions and analyses were performed at the Sequencing Core Facility, California Institute of Technology, on an ABI 373A automated sequencer (Applied Biosystems).

Construction of plasmids for mutation. pALS519b and pALS519f, pUC19 derivatives which have a 10 kb *Pst*I insert containing *mxhM* and *pqqAB(C/D)E* and whose orientation of the fragment is opposite to each other, were digested with *Bgl*II and ligated with the kanamycin-resistance gene cassette digested from pUC4K (Pharmacia) with *Bam*HI. The plasmids obtained were digested with *Pvu*II and *Xba*I and then ligated into the same restriction sites of the suicide vector pAYC61 (Chistoserdov *et al.*, 1994). The resultant plasmids were named pALS5Kan61b and pALS5Kan61f.

A pUC19 derivative, pHT1BH19, containing a 3.8 kb *Bam*HI–*Hind*III insert (see Fig. 1) was digested with *Pst*I and *Sac*II, treated with T4 DNA polymerase to make blunt ends, and then self-ligated. The resulting plasmid, pHT2SacH19, contains a 1.8 kb *Sac*II–*Hind*III fragment. pHT2SacH19 was digested with *Bgl*II and ligated with the *Bam*HI-digested kanamycin-resistance gene. The plasmid obtained was digested with *Pvu*II and *Xba*I and then ligated into the same restriction sites of pAYC61. The resultant plasmid was named pHT2SacHKan61. The construct was confirmed by restriction digest mapping and by sequencing across the ligation junctions.

Construction of plasmids for complementation. pHT1 was constructed with a 7.4 kb *Bam*HI–*Pst*I fragment from pALS519b, containing *mxhM* and *pqqAB(C/D)E*. pHT2, which has the *pqq* gene cluster but not *mxhM*, was constructed as follows. The 1.8 kb *Sac*II–*Hind*III fragment from pHT2SacH19 was isolated as a *Bam*HI–*Hind*III fragment and ligated into *Bam*HI/*Hind*III-digested pUC19 containing the 3.6 kb *Hind*III–*Pst*I fragment. The resultant 5.4 kb *Bam*HI–*Pst*I fragment was isolated and ligated into the same restriction sites of pRK310, a broad-host-range vector (Ditta *et al.*, 1985).

pHT3L was constructed as follows (see Fig. 2). pHT1BH19, a pUC19 derivative containing the 3.8 kb *Bam*HI–*Hind*III fragment, was digested with *Pfi*MI and *Nsi*I, which cut within *pqqA* and *pqqB*, respectively, removing a 0.1 kb *Pfi*MI–*Nsi*I fragment and an adjacent 0.2 kb *Nsi*I fragment. pHT3BH19 was then made by ligating the synthetic oligonucleotides shown in Fig. 2 to the *Pfi*MI/*Nsi*I-digested pHT1BH19. This eliminated all but the first dipeptide of *pqqA* and produced a deletion of 69 amino acids of *pqqB*. pHT3BH19 was digested with *Bam*HI and *Hind*III, and the 3.5 kb fragment containing *mxhM*pqqB'C' was ligated into the *Hind*III site of pUC19 with the 3.6 kb *Hind*III–*Pst*I fragment containing *pqqC/DE*. The resulting 7.1 kb *Bam*HI–*Pst*I fragment shown in Fig. 2 was isolated and ligated into the same restriction sites of pRK310. The resultant plasmid (pHT3L) contains *mxhM*, *pqq(C/D)E*, and a partial *pqqB*, which would produce PqqB with a 69 amino acid deletion in the N-terminus, immediately following the first two amino acids (Met His). pHT4L containing *mxhM* and *pqqB(C/D)E* was constructed as follows (see Fig. 2). pHT3BH19 was digested with *Nsi*I and the 0.2 kb *Nsi*I fragment from the 3.8 kb *Bam*HI–*Hind*III

Fig. 1. Schematic representation of plasmids used for complementation experiments. Thick lines show chromosomal DNA regions in each plasmid and thin lines are the deleted region. Open boxes at the top of the figure show the location of *mxhDM* and *pqq* genes; these genes are transcribed from left to right. Details of the construction of plasmids are given in Methods. Note that the *Sal*I and *Sac*II sites indicated are not unique in this *Pst*I fragment. GenBank accession numbers are L43136 for the 4.4 kb *Pst*I–*Bgl*II fragment, L25889 for the 2.0 kb *Bgl*II–*Hind*III fragment, and U72662 for the 3.6 kb *Hind*III–*Pst*I fragment.

Fig. 2. Schematic representation of the construction of pHT3L and pHT4L. The vector part of each plasmid is not shown. The synthetic oligonucleotides HT1 and HT2 were annealed and inserted into *Pst*I/*Nsi*I double-digested pHT3BH19. Detailed description of construction is given in Methods.

fragment was inserted. This *Nsi*I fragment contains the portion of *pqqB* deleted in pHT3BH19. The plasmid with correct orientation of the insert was selected by restriction mapping. The 3.7 kb *Bam*HI-*Hind*III fragment was isolated and ligated with the 3.6 kb *Hind*III-*Pst*I fragment, and the resulting 7.3 kb *Bam*HI-*Pst*I fragment was ligated into the same restriction sites of pRK310.

pHT3 was constructed as follows. A pUC derivative, pHT3SacH19, was constructed like pHT3BH19 as described above, except the starting fragment was the 1.8 kb *Sac*II-*Hind*III fragment. The *Sac*II-*Hind*III fragment containing the 0.3 kb deletion was isolated as a *Bam*HI-*Hind*III fragment and ligated with the 3.6 kb *Hind*III-*Pst*I fragment into pUC19. The 5.1 kb *Bam*HI-*Pst*I fragment was isolated and ligated into the same restriction sites of pRK310. pHT4 was made by taking advantage of the engineered *Clal* site in the oligonucleotide insertion in pHT4L (Fig. 2) and pHT3SacH19. The *Bam*HI-*Clal* fragment from pHT3SacH19 was combined with the *Clal*-*Hind*III from pHT4L, to generate a 1.7 kb *Bam*HI-*Hind*III fragment with a deleted *pqqA*, and this was combined with the 3.6 kb *Hind*III-*Pst*I fragment from pALS519b to generate the 5.3 kb insert containing *pqqB*(C/D)E. In the case of isolation of the fragments with *Clal* cohesive ends, plasmids isolated from *E. coli* GM2163 (*dam dcm*) were used.

The constructs were confirmed by restriction mapping and by DNA sequencing at the sites of deletion and ligation.

Mating. Plasmids of both pAYC61 and pRK310 derivatives were transferred to *M. extorquens* AM1 rif by using *E. coli* S17-1 as a donor host (Simon *et al.*, 1983). Mating was

performed on nitrocellulose filters on nutrient agar (Difco) for 20–24 h at 30 °C. Transconjugants were selected by rifamycin and kanamycin (for recombinant mutants) or tetracycline (for pRK310 derivatives) resistance on minimal medium containing succinate.

Determination of PQQ content. *M. extorquens* AM1 cells were grown on minimal medium for 48 h. Cells and culture supernatant were separated by centrifugation at 4000 *g* for 5 min at 4 °C. Cells were washed and suspended in 50 mM potassium phosphate buffer (pH 7.0) and crude extract was prepared as described previously (Toyama *et al.*, 1997). Crude extracts were pretreated at 100 °C for 5 min to release PQQ bound by MDH, prior to PQQ measurements.

The content of PQQ was determined spectrophotometrically as described previously (Toyama *et al.*, 1997), with the apo-form of soluble glucose dehydrogenase purified from the *E. coli* cells containing the cloned gene from *Acinetobacter calcoaceticus* (Cleton-Jansen *et al.*, 1989).

Hybridization with *pqqA* probe. The *pqqA* probe (Fig. 3) was tailed with fluorescein-11-dUTP, catalysed by terminal deoxynucleotidyl transferase (Amersham). Chromosomal DNA was isolated by the method of Marmur (1961). Chromosomal DNA (10 µg) digested with restriction enzyme(s) was separated by agarose gel electrophoresis, blotted onto a nylon membrane, hybridized with the probe, and detected using the ECL detection system (Amersham) with anti-fluorescein horseradish-peroxidase conjugate. Hybridization temperature was 58 °C and washing was done at 50 °C with 1 × SSC containing 0.1 % SDS.

RESULTS

Construction of a *pqqAB(C/D)E* deletion mutant

To study the phenotype of strains containing specific combinations of *pqq* genes, a mutant was constructed in which the *pqqAB(C/D)E* gene cluster was deleted. Plasmids were generated (pALS5Kan61b or pALS5Kan61f) in which the 4.1 kb *Bgl*II fragment containing *pqqAB(C/D)E* and part of *mxhM* (Fig. 1) had been removed and replaced with a kanamycin-resistance gene. No convenient restriction enzyme sites were available between *mxhM* and *pqqA*, and so this construction was used. *mxhM* is a gene required for transcription of several methanol oxidation genes, including the MDH subunits (Xu *et al.*, 1993; Springer *et al.*, 1995) and is suggested to be a response-regulator gene of a two-component regulatory system involving *mxhD*, a sensor kinase gene (Springer *et al.*, 1997). It is also involved in transcription of *pqqA* (Ramamoorthi & Lidstrom, 1995). pALS5Kan61b or pALS5Kan61f was introduced into *E. coli* S17-1 (*mob*-positive), transferred into *M. extorquens* AM1 rif, and transconjugants were selected on kanamycin and rifamycin and screened on tetracycline. One putative double-crossover insertion mutant (kanamycin-resistant, tetracycline-sensitive) was obtained with pALS5Kan61b and two were obtained with pALS5Kan61f. These three mutants were indistinguishable in carbon source utilization and complementation pattern with plasmids; therefore, only the results with one of them, 3b, are described hereafter.

Growth of the mutant and its transformants

Since *mxhM* is required for expression of the subunits of MDH (Xu *et al.*, 1993; Springer *et al.*, 1995), mutant 3b (with a defective *mxhM*) was unable to grow on

methanol even in the presence of PQQ. A series of plasmids containing different combinations of *mxhM* and *pqq* genes were constructed, and these were tested for complementation of mutant 3b (Fig. 1, Table 2). Wild-type growth of the mutant on methanol was restored with pHT1, containing *pqqAB(C/D)E* and *mxhM*. No growth was observed with plasmids that were missing *mxhM* (pHT2, pHT3, pHT4, pHT2Ns). Surprisingly, wild-type growth was observed by a strain with a plasmid that contained *mxhM* and *pqqB(C/D)E*, but not *pqqA*. The wild-type growth rate of this strain was confirmed in liquid culture. pHT4 was shown to contain functional *pqqB*, *pqqC* and *pqqE* genes by complementation of the appropriate *pqq* mutants (Table 2). These results indicate that the *pqqA* deletion did not cause a polar effect on downstream *pqq* genes. A plasmid (pHT3L) that contained *mxhM* and *pqq(C/D)E* with an in-frame N-terminal truncated form of *pqqB* allowed slow growth on methanol on agar plates but no growth in liquid culture. However, a similar response was observed with a *pqqB* mutant containing only the vector, suggesting that PqqB is not absolutely required for growth on methanol in *M. extorquens* AM1. PqqB has been suggested to be involved in the transport of PQQ or its intermediate from the cytoplasm to the periplasm (Velterop *et al.*, 1995).

PQQ production of the wild-type, the mutant and its transformants

PQQ production was examined in the wild-type, to determine the optimal assay conditions for assessing PQQ production in the mutants. The total PQQ produced by *M. extorquens* AM1 was measured in both crude extract and culture supernatant, and the majority (>95%) was found in the culture supernatant. The

Table 2. Growth on methanol medium of *pqq* mutants containing different plasmids

Growth was tested on minimal agar plates containing 0.5% methanol. —, No growth for 3 d; +, reduced growth compared to wild-type cells; ++, wild-type growth; NT, not tested.

Plasmid	Gene(s)	Growth of mutants:			
		3b [Δ <i>pqqAB(C/D)E</i> <i>mxhM</i>]	UV45 (<i>pqqE</i>)	EMS12 (<i>pqqC(D)</i>)	UV46 (<i>pqqB</i>)
pRK310	None	—	—	—	—*
pHT1	<i>mxhMpqqAB(C/D)E</i>	++	NT	NT	NT
pHT2	<i>pqqAB(C/D)E</i>	—	++	++	++
pHT3	<i>pqqB'(C/D)E</i>	—	++	++	—*
pHT3L	<i>mxhMpqqB'(C/D)E</i>	+	NT	NT	NT
pHT4	<i>pqqB(C/D)E</i>	—	++	++	++
pHT4L	<i>mxhMpqqB(C/D)E</i>	++	NT	NT	NT
pHT2Ns	<i>pqqA</i>	—	—	NT	—*
pHT2SacH	<i>pqqAB</i>	NT	NT	NT	++
pHT4SacH	<i>pqqB</i>	NT	NT	NT	++

* No growth for 3 d, but significant growth was observed after 1 week.

Fig. 3. PQQ amounts produced in the transformants: (a) in crude extract; (b) in total of crude extract and culture supernatant. PQQ amount was measured as described in Methods. Results are expressed as amounts per mg protein in the crude extract, to normalize all values to the amount of cells present. Open, black and hatched bars are the results of cultures grown with succinate, methanol plus methylamine, and methanol, respectively. Error bars show standard deviations of two to four experiments.

total PQQ produced was usually around 30 nmol (mg protein)⁻¹, which corresponded to about 10 μ M in the culture supernatant. When PQQ production was followed over time, it was found that PQQ appeared in the culture supernatant only after substantial growth had occurred. The amount of PQQ continued to increase as the culture entered stationary phase and then it plateaued. Therefore, PQQ production of the mutant and its transformants was measured in early stationary phase. Mutants and transformants that did not show growth on methanol were tested for PQQ production after 48 h incubation in the presence of methanol.

The amount of PQQ produced by the mutants was checked both in the culture supernatant and the crude extract in cells grown under three conditions, with succinate (non-inducing), with methanol (inducing, but no growth for some of the mutants), and with methanol plus methylamine (inducing, all strains grow) (Fig. 3). Crude extract was heat-treated to release the PQQ bound by MDH prior to assaying PQQ. In the wild-type, PQQ was detectable in crude extract prior to heat treatment, but it was only a trace amount as compared to the amount after heat treatment. PQQ amounts

detected in the crude extract were similar to that of wild-type for strains that could not produce MDH (mutant 3b with pHT2 or pHT4), and the amounts detected were similar before and after heat treatment of the crude extract. These results suggest that most of the PQQ in crude extract was bound to MDH in the wild-type. In all cases, PQQ was mainly found in the culture supernatant and only a small percentage of the total PQQ was in the crude extract, except in the case of the mutant 3b with pHT3L. This plasmid lacks *pqqAB*, and in this strain over half of the small amount of PQQ produced was found in the crude extract. This result supports the results observed in *K. pneumoniae*, suggesting that PqqB is involved in the transport of PQQ (Velterop *et al.*, 1995).

Maximum production of PQQ by the wild-type or its transformant with the vector pRK310 was observed when they grew on methanol, and about one-seventh that amount was produced by cells grown on succinate (Fig. 3). Intermediate levels were observed in cells grown on methanol plus methylamine. Neither the crude extract nor the culture supernatant of the mutant 3b with pRK310 contained detectable PQQ when grown under any of the three conditions (data not shown). The mutant with pHT1 produced PQQ in larger amounts than the wild-type under all three conditions, presumably as a result of the higher copy number of *pqq* genes. When the plasmid pHT2 (no *mxhM*) was introduced, succinate-grown cells produced about the same amount of PQQ as wild-type, but this amount did not increase significantly under inducing conditions. These results suggest that *mxhM* is essential for normal induction of PQQ production by methanol. The transformant with pHT4L (missing only *pqqA*) was able to grow on methanol and produced PQQ at levels about one-half to one-third that of wild-type cells, and one-fifth to one-tenth that of the deletion mutant containing pHT1. The PQQ production pattern in this strain was similar to the wild-type. The transformant containing pHT4 (no *mxhM* or *pqqA*) showed lower levels of PQQ as compared to the transformant containing pHT2 (no *mxhM*), and in keeping with the proposed role of *mxhM* in PQQ induction, this strain did not show methanol induction. In fact, the levels on methanol were lower than in the other two growth conditions. The mutant harbouring pHT3L (missing *pqqAB*) produced low but detectable amounts of PQQ in all conditions, and showed increased production of PQQ by cells transferred into medium containing methanol, although no growth was observed. No PQQ production was detected in the transformant with pHT3 (no *mxhM* or *pqqAB*), presumably reflecting accumulative defects in *pqqAB* and loss of induction by *mxhM*.

Construction of a *pqqA* insertion mutant and its properties

The data obtained with the large deletion mutant suggested that *pqqA* was not essential for PQQ production in *M. extorquens* AM1. To examine the role of the small peptide further, another mutant was isolated

<i>M. extorquens</i> AM1:	GTC	GGC	ATG	GAA	GTC	ACG	AGC	TAC	GAG
	V	G	M	E	V	T	S	Y	E
<i>P. fluorescens</i> :	ATC	GGC	TTC	GAA	GTC	ACC	ATG	TAC	CTT
	I	G	F	E	V	T	M	Y	F
<i>K. pneumoniae</i> :	CTC	GGT	CTG	GAA	GTG	ACG	CTG	TAC	ATT
	L	G	L	E	V	T	L	Y	I
<i>A. calcoaceticus</i> :	ATT	GGT	TTT	GAA	GTT	ACA	ATG	TAC	TTT
	I	G	F	E	V	T	M	Y	F
Probe:	GGC	ATG	GAA	GTC	ACG	AGC	TAC		
		T	C		G	C	CTG		
		C							

Fig. 4. The conserved region of known *pqqA* genes, with the probe used for detection of *pqqA*-like genes shown below.

by homologous recombination with pHT2SacHKan61, which has a kanamycin-resistance gene inserted in the *Bgl*II site in *pqqA*, between the start codon and the conserved region of PqqA shown in Fig. 4. Two putative double-crossover recombinants were isolated (kanamycin-resistant, tetracycline-sensitive). The double-crossover event was analysed for both recombinants by probing diagnostic digests of chromosomal DNA using an oligonucleotide probe targeted to the region immediately downstream of the insertion. The restriction enzymes used were chosen so that they cut within the kanamycin-resistance gene and downstream of the probed sequence. The hybridization revealed in each case that the wild-type bands had shifted to the predicted size, confirming the insertion event at the correct site within *pqqA*. These mutants were indistinguishable and one of them, D2, was used for further experiments. The mutant D2 grew on agar plates containing methanol as well as the wild-type cells, and the maximum growth rate in liquid culture was similar to that of the wild-type cells grown on succinate or methanol. However, total PQQ production was only 5–11% of the wild-type in a 48 h culture (Table 3). Most of this decrease was in the culture supernatant fraction, as the PQQ in the crude extract was still about half that found for the wild-type cells. A time course of PQQ production by methanol-grown D2 cells showed that PQQ eventually reached about 4 µM in the culture supernatant when cells were

Fig. 5. PQQ production of the mutant D2 and its transformants. Cells were grown on succinate and transferred to the same volume of fresh medium containing methanol at time zero. The PQQ amount in each culture supernatant was measured as described in Methods. Symbols represent transformants of the mutant D2 with pRK310 (○), pHT2Ns (×), pHT2SacH (□), pHT2 (▲) and pHT4 (■).

incubated longer after entering stationary phase (Fig. 5). This stationary-phase culture was diluted and spread on agar medium with either methanol alone or succinate plus kanamycin. The cell numbers on both agar plates were similar, indicating that no significant levels of kanamycin-sensitive revertant cells were present in the culture.

Plasmids containing *pqqA* were transferred into the D2 mutant, and PQQ produced in the culture medium was measured (Fig. 5). Both pHT2Ns, which contains only *pqqA* and pHT2SacH, which contains *pqqAB*, had little effect on PQQ production. pHT2 increased PQQ production to that of the wild-type cells or more (Fig. 5). Failure to complement the *pqqA* mutant with pHT2Ns and pHT2SacH might be explained by polarity from the kanamycin-resistance gene insertion. However, pHT4, which contains *pqqB(C/D)E* but not *pqqA*, increased PQQ production only slightly.

Table 3. PQQ produced into the culture supernatant by the mutant D2 grown on different substrates

PQQ amounts were measured as described in Methods, and normalized to the amount of protein in the crude extract.

	Amount of PQQ [nmol (mg protein) ⁻¹] in cells grown on:		
	Succinate	Methanol + methylamine	Methanol
In culture supernatant	(97.1 ± 31.5)* 0.441 ± 0.17	(364 ± 245)* 1.42 ± 1.2	(610 ± 450)* 1.81 ± 1.24
In crude extract	0.18 ± 0.05	0.29 ± 0.09	0.69 ± 0.26
Total amount produced	0.59 ± 0.12	1.71 ± 1.11	2.50 ± 0.98

* Values in parentheses are nM.

Detection of genes similar to *pqqA*

It is possible that a second *pqqA* or a different gene that produces a peptide similar to PqqA exists in *M. extorquens* AM1. If so, this would explain why the *pqqA* mutants are able to produce sufficient amounts of PQQ for normal growth on methanol. Hybridization experiments were carried out to search for *pqqA*-like genes in the DNA of *M. extorquens* AM1. The probe was designed to take into account the conserved amino acids present in the known PqqA peptides and the codon bias of *M. extorquens* AM1 (Fig. 4). This probe showed only one signal, a 4.5 kb *SalI* fragment or a 3.8 kb *BamHI*–*HindIII* fragment in digested genomic DNA from wild-type cells, the fragments known to contain *pqqA*. No signals were detected in the digested genomic DNA of the mutant 3b or for the plasmid pHT4L, confirming the lack of *pqqA* or a *pqqA*-like gene in these cases. Further experiments at lower stringencies also failed to identify a second band with the *pqqA* probe.

DISCUSSION

A mutant of *M. extorquens* AM1 lacking the *pqq* gene cluster *pqqAB(C/D)E* was obtained and analysed by complementation. Unexpectedly, strains complemented with a plasmid containing *pqqB(C/D)E* but not *pqqA* grew well on methanol, with a PQQ production of 10–20% that of the wild-type containing the entire *pqq* gene cluster on a plasmid. This phenotype was confirmed with another mutant, in which a kanamycin-resistance gene was inserted within *pqqA* in the chromosome. This mutant also grew well on methanol, even though its PQQ production was about 10–20% that of the wild-type. However, the crude extract of this mutant contained PQQ amounts about half that of the wild-type. Since the majority of the PQQ in crude extracts appears to be found in MDH, these data suggest that the reduced production of PQQ in the mutant was still sufficient to generate enough active MDH to support normal growth of the mutant on methanol. Therefore, disruption of *pqqA* reduced production of PQQ, but did not abolish it.

These results are surprising, because several lines of evidence suggest that the small peptide encoded by *pqqA* is directly involved in the formation of PQQ. In *A. calcoaceticus*, site-directed mutagenesis at the conserved tyrosine or glutamate residue abolished the production of PQQ (Goosen *et al.*, 1992), and a frame-shift mutation in *pqqA* of *K. pneumoniae* also abolished PQQ synthesis (Meulenbergh *et al.*, 1992). The latter mutation could be complemented *in trans* (Velterop *et al.*, 1995), suggesting that the translated peptide itself might be involved in PQQ synthesis. PqqF of *M. extorquens* AM1 and *K. pneumoniae* show identity to the mitochondrial processing peptidase family and PqqG of *M. extorquens* AM1 shows identity to the pitrilysin/insulin-degrading enzyme family (Springer *et al.*, 1996), implying that they could cleave the peptide or its derivative in the course of PQQ synthesis. In addition, the steady-state level of *pqqA* mRNA is very high compared to mRNA for the

rest of the gene cluster, as would be expected for a precursor, and the predicted ribosome-binding site of *pqqA* is very strong while those of the other *pqq* genes are weak, which could further enhance the levels of PqqA over the other *pqq* gene products (Ramamoorthi & Lidstrom, 1995). All of these data support the hypothesis that the PqqA peptide is the precursor of PQQ. It is possible that a peptide similar to PqqA is present in *M. extorquens* AM1 that can take the place of PqqA in PQQ synthesis, but an attempt to find such a gene failed with a probe based on conserved *pqqA* DNA sequences available from four bacteria. We cannot exclude the possibility that a peptide encoded by a gene that was not detected in the hybridization experiment is used for PQQ synthesis in the *pqqA* mutants. Alternatively, it is possible that something other than the peptide might be involved in PQQ synthesis, either as the normal synthesis pathway or as a less efficient route only revealed in the PqqA mutant. For instance, it is possible that in *M. extorquens* AM1 free tyrosine and glutamate might generate PQQ with lower efficiency than PqqA. Whatever this alternative pathway might be, it requires all of the other *pqq* genes. Our results suggest that an *in vitro* system for producing PQQ using a labelled peptide will be necessary to determine whether the peptide can be the precursor of PQQ.

Our results also demonstrate that *mxhM* is required for normal methanol induction of PQQ synthesis. Previous results had shown that MxbM has similarity to a family of response-regulator proteins, and together with MxbD, apparently forms a sensor–regulator pair involved in transcription of a number of methanol oxidation genes including *pqqA* (Ramamoorthi *et al.*, 1995; Springer *et al.*, 1997). We have now shown that even in strains lacking *pqqA*, the production of PQQ shows the normal regulatory pattern if *mxhM* is present, but not if this gene is absent. These results show that whatever the route of PQQ synthesis in the *pqqA* mutants may be, it is methanol-inducible and this regulation is mediated by the *mxhM* system.

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