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Synthesis of Pyrroloquinoline Quinone In Vivo and In Vitro and Detection of an Intermediate in the Biosynthetic Pathway

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In Klebsiella pneumoniae, six genes, constituting the pqqABCDEF operon, which are required for the synthesis of the cofactor pyrroloquinoline quinone (PQQ) have been identified. The role of each of these K. pneumoniae pqq genes was examined by expression of the cloned pqq genes in Escherichia coli, which cannot synthesize PQQ. All six pqq genes were required for PQQ biosynthesis and excretion into the medium in sufficient amounts to allow growth of E. coli on glucose via the PQQ-dependent glucose dehydrogenase. Mutants lacking the PqqB or PqqF protein synthesized small amounts of PQQ, however. PQQ synthesis was also studied in cell extracts. Extracts made from cells containing all Pqq proteins contained PQQ. Lack of each of the Pqq proteins except PqqB resulted in the absence of PQQ. Extracts lacking PqqB synthesized PQQ slowly. Complementation studies with extracts containing different Pqq proteins showed that an extract lacking PqqC synthesized an intermediate which was also detected in the culture medium of pqqC mutants. It is proposed that PqqC catalyzes the last step in PQQ biosynthesis. Studies with cells lacking PqqB suggest that the same intermediate might be accumulated in these mutants. By using pqq-lacZ protein fusions, it was shown that the expression of the putative precursor of PQQ, the small PqqA polypeptide, was much higher than that of the other Pqq proteins. Synthesis of PQQ most likely requires molecular oxygen, since PQQ was not synthesized under anaerobic conditions, although the pqq genes were expressed.

Pyrrloquinoline quinone (PQQ) is a cofactor of several bacterial dehydrogenases and transfers reductive equivalents to the respiratory chain. The physiological electron acceptors vary from ubiquinone in the case of membrane-bound glucose dehydrogenase (e.g., glucose dehydrogenase of Acinetobacter calcoaceticus) to a cytochrome c in the case of methanol dehydrogenases (e.g., methanol dehydrogenase of Methylococcus extorquens AM1) for a review, see reference 2). The chemical structure of PQQ has been determined (13, 33), but the biosynthetic pathway of PQQ has not yet been solved. From 13C nuclear magnetic resonance studies with Hyphomicrobium X and M. extorquens AM1, it was suggested that the amino acids tyrosine and glutamic acid are the precursors for PQQ (19, 44). Studies to detect intermediates in PQQ biosynthesis in A. calcoaceticus, Methylobacterium organophilum, and Pseudomonas aureofaciens have been negative thus far (43).

Genes involved in PQQ biosynthesis have been cloned from several organisms. Five A. calcoaceticus pqq genes, pqqIV, V, I, II, and III (15, 17), and six K. pneumoniae pqq genes, pqqA, B, C, D, E, and F (25, 26), were cloned and sequenced. Comparison of the deduced amino acid sequences showed that the proteins encoded by the first five genes of the K. pneumoniae pqq operon (pqqABCDE) show similarity to the proteins encoded by the corresponding A. calcoaceticus genes (49 to 64% identical amino acid residues). The K. pneumoniae pqqF gene encodes a protein that shows similarity to Escherichia coli protease III and other proteases (26), but its equivalent has not yet been found in A. calcoaceticus. Recently, three M. extorquens AM1 pqq genes, pqqD, G, and H, have been cloned and sequenced (28); pqqC was only partly sequenced. The encoded proteins showed similarity to the K. pneumoniae PqqA, B, and C proteins and the A. calcoaceticus PqqIV, V, and I proteins, respectively. Four additional pqq genes have been detected in M. extorquens by isolation of mutants and complementation studies. From similar studies, six (possibly seven) pqq genes have been postulated in M. organophilum DSM760 (4). Finally, a DNA fragment cloned from Erwinia herbicola contained a gene encoding a protein similar to K. pneumoniae PqqE and A. calcoaceticus PqqI(III). Except for the K. pneumoniae PqqF protein, none of the Pqq proteins shows similarity to other proteins in the database.

One of the pqq genes is small and may encode a polypeptide of 24 amino acids (PqqIV, A. calcoaceticus), 23 amino acids (PqqA, K. pneumoniae), or 29 amino acids (PqqD, M. extorquens AM1). Interestingly, these putative polypeptides contain conserved glutamate and tyrosine residues (positions 15 and 19, respectively, in K. pneumoniae and the equivalents in A. calcoaceticus and M. extorquens). Those residues have been suggested previously as precursors in PQQ biosynthesis. Replacement of Glu-16 by Asp and Tyr-20 by Phe in A. calcoaceticus PqqIV abolished PQQ biosynthesis (16). A frameshift in K. pneumoniae pqqA had the same result (26). It was suggested that the PqqA/PqqIV polypeptide might act as a precursor in PQQ biosynthesis (15, 16, 26).

Our aim is to elucidate the route of PQQ biosynthesis and the role of each of the six known K. pneumoniae pqq genes in this process. We have taken advantage of the fact that E. coli is unable to synthesize and excrete PQQ unless supplied with the six K. pneumoniae pqq genes (25, 26). Using plasmids in which one of the six pqq genes is inactivated at the time, we have investigated PQQ synthesis in vivo and in vitro. We also

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examined the expression of the different pqq genes of *K. pneumoniae*, especially *pqqA*.

(Part of this work was presented in a preliminary form at the 9th Meeting on Vitamin B₆ and Carbonyl Catalysis and the 3rd Meeting of PQQ and Quinoproteins at Capri, 22–27 May 1994.)

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth media.** The bacterial strains, plasmids, and growth media used in this study are listed in Table 1. The growth media used were Luria broth (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl in demineralized water [pH 7]) and minimal medium A (36) supplemented with 0.4% gluconate and the required amino acids and vitamins (25 µg/ml). Ampi-

<table>
<thead>
<tr>
<th>Strain, phage, or plasmid</th>
<th>Relevant genotype or properties</th>
<th>Source or reference</th>
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<tr>
<td>ED8654</td>
<td>supE supF metB ton⁺ hsdR</td>
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<td>KA196</td>
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<td>lacZ100: Tn10 minitet pqq-18: Tn3lacZ</td>
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<td>lacZ100: Tn10 minitet pqqB24: Tn5lacZ</td>
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<td>ptsI103 pqqB38: Tn5lacI</td>
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<td>KA222</td>
<td>ptsI103 pqqC40: Tn5lacI</td>
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<td>Tn5lacI</td>
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<td><strong>Plasmids</strong></td>
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<tr>
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<tr>
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<tr>
<td>pRE1</td>
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<td>pBR322</td>
<td>Ap⁺ Te⁺</td>
<td>Cloning vector</td>
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Abbreviations: P1, phage P1; Cm, chloramphenicol; Ap, ampicillin; Te, tetracycline; Km, kanamycin; Hyb, hybrid; mcs, multiple cloning site; rbs, ribosome-binding site.
cillin and kanamycin were used at 50 μg/mL, chloramphenicol was used at 34 μg/mL, and tetracycline was used at 20 μg/mL. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used as an inducer when pqq genes were placed under control of the lac promoter and to induce T7 RNA polymerase, which was under control of the lacUV5 operator. The methods used for preparing cells for λ phage stocks and λ phage DNA were described by Schleif (33). Transformation, digestion, and ligation were performed by standard procedures (34). Restriction and modification enzymes and buffers were obtained from Pharmacia, Bioray, and Gibco BRL. Plasmid DNA was isolated by the alkaline lysis method (34). For large-scale DNA isolations, RNA was removed by LiCl precipitation followed by RNAse treatment (29).

Construction of K. pneumoniae KA196, KA220, and KA222. To isolate a Tn10 insertion in the K. pneumoniae lacZ gene, K. pneumoniae NCTC418 was made sensitive to spectinomycin by the introduction of plasmid pAMH62 and then infected with ANK1098, as described by Way et al. (45). White colonies were selected on Luria agar plates containing tetracycline, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/mL), and IPTG (40 μg/mL). The β-galactosidase activity of one of these mutants, KA196, was reduced to background levels.

A K. pneumoniae strain defective in only pqqD or pqqC was constructed by transferring the pqqB::lacZu1C1 allele (from pBCP272) or the pqqC::lacZu1C1 allele (from pBCP274) to the chromosome of K. pneumoniae KA256 as described elsewhere (24). The resulting strains were designated KA220 and KA222, respectively.

Construction of pqq-lacZ operon fusions. Several pqq-lacZ operon fusions were constructed by deleting E. coli W3350/pBCP138 with λko2 (containing the lacZ operon and a promoterless lacZ gene in the left-end inserted repeat of Tn5 [JS59L] [37]) for 30 min at 37°C and selecting kanamycin- and chloramphenicol-resistant colonies on Luria agar plates. Plasmid DNA from the pooled mutants was transferred into E. coli W3350/pBCP169, and blue transformants were selected on Luria agar plates containing kanamycin, chloramphenicol, and X-Gal. The location of the Tn5 insertion was determined by restriction analysis. The fusions were transferred to the chromosome of KA196 as described elsewhere (24), yielding KA197 (pqq-1S-lacZu1: insertion between pqqA and pqqB), KA204 (pqqB34::lacZu1: insertion in the middle of pqqE), and KA205 (pqqB22::lacZu1: insertion in the middle of pqqF) (Fig. 1A). The exact positions of the lacZ fusions in KA197 (98 bp downstream of the pqqA start codon) in the pqqA-pqqB intercistronic space) and KA204 (340 bp downstream of the pqqA start codon) were determined by sequencing the fusion points.

Construction of plasmids. (i) Plasmids with an incomplete set of genes. Nonpolar insertions of the Tn5lac1 element (9) in the pqqB and pqqC genes of pBCP138 were isolated by infection of E. coli W3350/pBCP138 with λko2 (containing the Tn5 with a promoterless lacZ gene in the left-end inserted repeat of Tn5 [JS59L] [37]) for 30 min at 37°C and selecting kanamycin- and chloramphenicol-resistant colonies on Luria agar plates. Plasmid DNA from the pooled mutants was transferred into E. coli MC1060, and blue transformants were selected on Luria agar plates containing kanamycin, chloramphenicol, and X-Gal. The location of the Tn5 insertion was determined by restriction analysis. The fusions were transferred to the chromosome of KA196 as described elsewhere (24), yielding KA197 (pqq-1S-lacZu1: insertion between pqqA and pqqB), KA204 (pqqB34::lacZu1: insertion in the middle of pqqE), and KA205 (pqqB22::lacZu1: insertion in the middle of pqqF) (Fig. 1A). The exact positions of the lacZ fusions in KA197 (98 bp downstream of the pqqA start codon) in the pqqA-pqqB intercistronic space) and KA204 (340 bp downstream of the pqqA start codon) were determined by sequencing the fusion points.

Preparation of cell extracts. To prepare cell extracts to study in vitro synthesis of PQQ, E. coli JA221 cells containing one or more plasmid-borne pqq genes were grown overnight at 37°C in minimal medium A containing glucose and centrifuged at 10,000 × g for 10 min. The pellet was washed twice with 0.5% (vol/vol) of 1,4-piperazinediethanesulfonic acid (PIPES) in water, pH 7.0, containing 0.5 mM EDTA. The pellets were resuspended in 1 mM MgCl2, 100 mM PIPES, pH 7.0, and the cell extracts were centrifuged at 10,000 × g for 10 min. The supernatant was collected, and the cell pellets were resuspended in 100 mM 1,4-piperazinediethanesulfonic acid (PIPES) in water, pH 7.0.

To construct a plasmid with a lacZ fusion in pqqB, pBCP168 was digested with SalI and XhoI, and the SalI-XhoI fragment was inserted into SalI-XhoI-digested pET28b (+) (Ambion) to create pET28b (+)-pqqB. The fragment was sequenced to confirm that no additional mutations had occurred and cloned into E. coli JM109 electrocompetent cells. The resulting plasmid, pBCP361, was digested with SalI and XhoI, and the SalI-XhoI fragment was inserted into SalI-XhoI-digested pET3a (+) (Ambion) to create pET3a (+)-pqqB. The fragment was sequenced to confirm that no additional mutations had occurred and cloned into E. coli JM109 electrocompetent cells. The resulting plasmid, pBCP362, was digested with SalI and XhoI, and the SalI-XhoI fragment was inserted into SalI-XhoI-digested pET3b (+) (Ambion) digested with NdeI and KpnI. The resulting plasmid, pBCP364, was under the control of the T7 promoter.

Preparation of cell extracts. To prepare cell extracts to study in vitro synthesis of PQQ, E. coli JA221 cells containing one or more plasmid-borne pqq genes were grown overnight at 37°C in minimal medium A containing glucose and centrifuged at 10,000 × g for 10 min. The pellet was washed twice with 0.5% (vol/vol) of 1,4-piperazinediethanesulfonic acid (PIPES) in water, pH 7.0, containing 0.5 mM EDTA. The pellets were resuspended in 1 mM MgCl2, 100 mM PIPES, pH 7.0, and the cell extracts were centrifuged at 10,000 × g for 10 min. The supernatant was collected, and the cell pellets were resuspended in 100 mM 1,4-piperazinediethanesulfonic acid (PIPES) in water, pH 7.0.
FIG. 1. Schematic representation of pqq-lacZ fusions and pqq plasmids. Details of the construction of strains and plasmids are given in Materials and Methods. The pqq genes, in particular pqqA, are not drawn completely to scale. Ptac, lac promoter; Pppq, pqq promoter. Symbols: ▽, Tn5lacZ element; ψ, EcoRI linker; ∨, Tn5lac1 element; △, deletion; ▼, Tn5Km element. (A) pqq-lacZ operon fusions on the K. pneumoniae chromosome. (B) pqq-lacZ plasmid-borne protein fusions. (C) Plasmids containing various pqq genes. Numbering is for K. pneumoniae KA strains (A) or for pBCP plasmids (band C).
dehydrogenase (apo-GCD) or apo-ethanol dehydrogenase (apo-EDH) (see below).

**PQQ assay.** PQQ was determined with two different apo-enzymes, the soluble apo-GCD of *A. calcoaceticus* (gift from A. J. J. Osthoff, Delft University of Technology) and apo-EDH from *Comamonas testosteroni* (gift from G. A. H. de Jong, Delft University of Technology) (12). The assays were slight modifications of those described elsewhere (42) and allowed the determination of PQQ concentrations in the range of 0.6 to 15 nM (apo-GCD) and 2 to 50 nM (apo-EDH). A calibration curve was made with PQQ (Fluka) dissolved in minimal medium A. For cell extracts, the amount of PQQ was expressed as picomoles per milligram of protein. As a consequence, the detection level was 0.4 pmol/mg of protein with apo-GCD and 1.3 pmol/mg of protein with apo-EDH.

(i) GCD assay. First, the sample (50 μl) was mixed with 120 μl of 0.1 M Tris-HCl (pH 7.5) containing 3 mM CaCl₂ and 0.02 μM apo-GCD and incubated for 5 to 15 min at room temperature. Then, a 0.1 M Tris-HCl (pH 7.5) solution containing 3 mM CaCl₂, 1.2 mM phenazine methosulfate, and 0.063 mM 2,6-dichlorophenolindophenol was added to give a total volume of 950 μl. The reaction was started by adding 50 μl of 1 M glucose in demineralized water, and the decrease in absorbance at 600 nm was measured.

(ii) EDH assay. PQQ was determined with apo-EDH on a Cobas Bio automatic analyzer (Hoffmann-La Roche). The sample (80 μl) and demineralized water (15 μl) were mixed with 80 μl of 0.1 M Tris-HCl (pH 7.5) containing 1 mM apo-EDH and 5 mM CaCl₂. After incubation for 10 min at 25°C, the reaction was started by adding 80 μl of a solution containing 48 mM Tris-HCl (pH 7.5), 0.4 mM 2,6-dichlorophenolindophenol, and 1.5 mM Wursters Blue. The decrease in absorbance at 600 nm was measured and corrected for reduction of Wurters Blue in the absence of EDH.

**Assay for intermediate in PQQ biosynthesis.** To determine the amount of intermediate in the PQQ biosynthesis, 50 μl of sample (culture supernatant or supernatant from cell extract) was mixed with 480 mM Tris-HCl (pH 7.5), 100 μM apo-GCD, and 3 mM CaCl₂. After incubation for 30 min at 37°C with shaking, the mixture was transferred to a 1-ml cuvette, and the assay was continued as described above for the PQQ assay. To correct for possible PQQ already present, the sample was also assayed for PQQ with apo-GCD.

**β-Galactosidase activity.** β-Galactosidase activity was measured as described elsewhere (27), and the activity was expressed as nanomoles of o-nitrophenyl-$β$-D-galactopyranoside (ONPG) hydrolyzed per minute per milliliter of cells (optical density at 600 nm in 1). The molecular mass of protein standards are indicated on the left.

**Protein determination.** The amount of protein was determined with the bicinchoninic acid (Sigma) method (39). The sample was carried out according to the instructions of the manufacturers, on a Cobas Bio automatic analyzer (Hoffmann-La Roche), with bovine serum albumin as a standard.

## RESULTS

**Expression of pqq-lacZ operon fusions.** The expression of the *K. pneumoniae* *pqq* operon was studied with the help of several chromosomal *pqq-lacZ* operon fusions (see Fig. 1A). Cells were grown in LB and harvested at the exponential phase. Table 2 shows that the fusions located close to the *pqq* promoter had a higher β-galactosidase activity than the fusions further downstream. The highest β-galactosidase activity, that of the *pqq-lacZ* fusion located in the intercistronic space between *pqqA* and *pqqB* (KA197), was 15-fold lower than the induced wild-type β-galactosidase activity in *K. pneumoniae* NCTC418.

**PQQ synthesis under aerobic and anaerobic conditions.** To investigate the role of molecular oxygen in PQQ biosynthesis, we measured PQQ production under aerobic and anaerobic culture conditions. To switch the culture to anaerobic conditions, the cells were diluted 1:50 into fresh medium and flushed with N₂ for 30 min. The PQQ level in a wild-type *K. pneumoniae* strain is low and close to the detection level, we used wild-type *K. pneumoniae* NCTC418 harboring pBCP165, containing the complete *pqq* operon. Under anaerobic conditions, little PQQ was detected in the culture supernatant (12 nM) compared with aerobic conditions (540 nM). The small amount of PQQ detected under anaerobic conditions could be derived from the (aerobically grown) precursor.

Since the failure to synthesize PQQ under anaerobic conditions could be due to the lack of expression of the *pqq* genes, two chromosomal *pqq-lacZ* operon fusions were investigated. However, anaerobiosis had no significant effect on the β-galactosidase activity in KA197 (*pqq::Tn5lacZ*) and KA204 (*pqqB24::Tn5lacZ*) (Table 2).

**Expression of *pqqA*.** To investigate whether the *pqqA* gene encoded a polypeptide, the *pqqA* gene was cloned behind the strong, inducible T7 promoter. *E. coli* BL21(DE3) cells containing the resulting plasmid, pBCP364, produced a polypeptide of the size predicted for PqqA (2.7 kDa) upon induction with IPTG, whereas uninduced cells did not produce such a polypeptide (Fig. 2).

If PqqA is the precursor for PQQ biosynthesis, it would be

![FIG. 2. Synthesis of the *pqqA* gene product. *E. coli* BL21(DE3) cells carrying pBCP364 with *pqqA* cloned behind the T7 promoter (pBCP364) were grown to an optical density at 600 nm of 0.8 in the presence or absence of IPTG. Total cell protein was analyzed by tricine-SDS-PAGE as described in Materials and Methods, followed by Coomassie blue staining. Lane 1, no IPTG; lane 2, induction for 1 h with 400 μM IPTG. The molecular masses of protein standards are indicated on the left.](image-url)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fusion</th>
<th>β-Galactosidase activity (nmol of ONPG hydrolyzed/min/ml of cell culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA196</td>
<td>None</td>
<td>1.2 1.2 0.9</td>
</tr>
<tr>
<td>KA197</td>
<td>Between <em>pqqA</em> and <em>pqqB</em></td>
<td>10 12.5 10.2</td>
</tr>
<tr>
<td>KA204</td>
<td><em>pqqB-lacZ</em></td>
<td>4.8 7.0 6.1</td>
</tr>
<tr>
<td>KA202</td>
<td><em>pqqA-lacZ</em></td>
<td>1.5 ND ND</td>
</tr>
<tr>
<td>NCTC418</td>
<td>None; wild-type lacZ</td>
<td>146 ND ND</td>
</tr>
</tbody>
</table>

*a* Cells were grown in batch culture in LB or in minimal medium A containing 0.4% glucose (MM) and harvested in the exponential phase.

*b* ND, not determined.

*c* After induction with 1 mg IPTG.

### TABLE 2. Expression of *pqq-Tn5lacZ* operon fusions on the chromosome of *K. pneumoniae*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fusion</th>
<th>β-Galactosidase activity (nmol of ONPG hydrolyzed/min/ml of cell culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA196</td>
<td>None</td>
<td>1.2 1.2 0.9</td>
</tr>
<tr>
<td>KA197</td>
<td>Between <em>pqqA</em> and <em>pqqB</em></td>
<td>10 12.5 10.2</td>
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<td>KA204</td>
<td><em>pqqB-lacZ</em></td>
<td>4.8 7.0 6.1</td>
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<tr>
<td>KA202</td>
<td><em>pqqA-lacZ</em></td>
<td>1.5 ND ND</td>
</tr>
<tr>
<td>NCTC418</td>
<td>None; wild-type lacZ</td>
<td>146 ND ND</td>
</tr>
</tbody>
</table>

*a* Cells were grown in batch culture in LB or in minimal medium A containing 0.4% glucose (MM) and harvested in the exponential phase.

*b* ND, not determined.

*c* After induction with 1 mg IPTG.
expected that \textit{pqqA} would encode a polypeptide which is produced in higher amounts than the other \textit{Pqq} proteins. To compare the expression of the different \textit{pqq} genes, \textit{lacZ} fusions were constructed with \textit{pqqA} (pBCP361), \textit{pqqC} (pBCP362), and \textit{pqqE} (pBCP363) (see Fig. 1B). The activity of the fusion proteins was measured in \textit{E. coli} MC1060. The \textit{lacZ} fusion in \textit{pqqA} resulted in a 20-fold-higher β-galactosidase activity (500 nmol of ONPG/min/ml of culture) than the \textit{lacZ} fusions in \textit{pqqC} and \textit{pqqE} (23 and 19 nmol of ONPG/min/ml of culture, respectively).

### In vivo complementation and growth studies

In vivo complementation studies were used to investigate whether all six \textit{pqq} genes were necessary for \textit{PQQ} production and excretion and to test the functionality of the plasmids used in this study. All plasmids used are shown in Fig. 1C. For in vivo complementation, two compatible plasmids, each containing an incomplete set of \textit{pqq} genes, were transformed together into the \textit{E. coli recA} strain JA221. As a control, each of the plasmids was transformed separately. The cells containing the various \textit{pqq} plasmids were grown overnight in minimal medium containing gluconate, and \textit{PQQ} was measured in the culture supernatant. Table 3 shows that all plasmid combinations in which at least one copy of each of the six \textit{pqq} genes was present resulted in \textit{PQQ} synthesis and excretion. No \textit{PQQ} was detected in supernatants from cell cultures harboring only a single plasmid which lacked either \textit{pqqA}, \textit{C}, \textit{D}, or \textit{E}. In supernatants of cell cultures harboring plasmids lacking \textit{pqqB} (pBCP324 and pBCP328) or \textit{pqqF} (pBCP186 and pBCP499), small amounts of \textit{PQQ}, only slightly above the detection level, were measured (Table 3).

To study whether all \textit{pqq} genes are required for \textit{growth} on glucose minimal medium via glucose dehydrogenase, we transformed \textit{E. coli} ZSC112, which is unable to grow on glucose because of a \textit{ptsM} and \textit{ptsG} mutation, with various plasmids lacking one of the six \textit{pqq} genes. Growth on glucose was not stimulated by any of these plasmids, whereas the control plasmid pBCP165 (\textit{pqqABCDEF}) stimulated growth (Table 3).

### In vitro \textit{PQQ} synthesis

The role of the various \textit{Pqq} proteins in \textit{PQQ} biosynthesis was studied with the help of an in vitro system in which a cell extract containing all but one of the \textit{Pqq} proteins was combined with an extract containing the missing \textit{Pqq} protein. All plasmids used for the in vitro studies are shown in Fig. 1C. The presence of \textit{PQQ} was detected with two different apo-enzymes specific for \textit{PQQ}, apo-GCD and apo-EDH. The \textit{PQQ} values determined with apo-GCD and apo-EDH agreed. A cell extract lacking all six \textit{Pqq} proteins contained less than 0.4 pmol of \textit{PQQ} per mg of protein, whereas a cell extract with all six \textit{Pqq} proteins, \textit{PqqA}, \textit{B}, \textit{C}, \textit{D}, \textit{E} and \textit{F}, contained approximately 12 pmol of \textit{PQQ} per mg of protein. In the latter case, the amount of \textit{PQQ} did not increase with prolonged incubation (Table 4 and Fig. 3A). The intracellular \textit{PQQ} concentration was calculated to be approximately 3.5

<table>
<thead>
<tr>
<th>\textit{pqq} genes</th>
<th>Plasmid(s)</th>
<th>IPTG</th>
<th>\textit{PQQ} concn (nM)</th>
<th>Growth on glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
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<td>&lt;0.6</td>
<td>−</td>
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<tr>
<td>\textit{ABCD}</td>
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<td>180</td>
<td>+</td>
<td>+</td>
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<tr>
<td>\textit{Complementation with pqqA}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−BCDEF + \textit{A}</td>
<td>pBCP325 + pBCP335</td>
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<td>−</td>
<td>−</td>
</tr>
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<td>−</td>
</tr>
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<td>\textit{A}</td>
<td>pBCP335</td>
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<td>−</td>
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<td>\textit{Complementation with pqqB}</td>
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</tr>
<tr>
<td>\textit{A}−\textit{CDEF} + \textit{AB}</td>
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<td>0.6</td>
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<td>pBCP332</td>
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<td>−</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABC}</td>
<td>pBCP332</td>
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<td>−</td>
<td>−</td>
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<tr>
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<tr>
<td>\textit{AB}−\textit{DEF} + \textit{ABC}</td>
<td>pBCP329 + pBCP337</td>
<td>144</td>
<td>+</td>
<td>−</td>
</tr>
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<td>\textit{AB}−\textit{DEF} + \textit{C}</td>
<td>pBCP329 + pBCP390</td>
<td>30</td>
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<td>−</td>
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<td>\textit{AB}−\textit{DEF}</td>
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<td>&lt;0.6</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABC}</td>
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<td>&lt;0.6</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABC}</td>
<td>pBCP337</td>
<td>&lt;0.6</td>
<td>−</td>
<td>−</td>
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<tr>
<td>\textit{Complementation with pqqD}</td>
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<tr>
<td>\textit{ABC}−\textit{EF} + \textit{ABCD}</td>
<td>pBCP328 + pBCP341</td>
<td>48</td>
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<td>−</td>
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<td>\textit{ABC}−\textit{EF}</td>
<td>pBCP328</td>
<td>+</td>
<td>&lt;0.6</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABCD}</td>
<td>pBCP328</td>
<td>&lt;0.6</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABCD}</td>
<td>pBCP328</td>
<td>&lt;0.6</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>\textit{Complementation with pqqE or pqqF}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{ABCD}−\textit{D} + \textit{ABCDE}</td>
<td>pBCP329 + pBCP186</td>
<td>96</td>
<td>+</td>
<td>−</td>
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<tr>
<td>\textit{ABCD}−\textit{D} + \textit{BCDEF}</td>
<td>pBCP329 + pBCP325</td>
<td>11</td>
<td>+</td>
<td>−</td>
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<tr>
<td>\textit{ABCD}−\textit{D}</td>
<td>pBCP329</td>
<td>+</td>
<td>&lt;0.6</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABCD}</td>
<td>pBCP330</td>
<td>0.6</td>
<td>−</td>
<td>−</td>
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<tr>
<td>\textit{ABCD}</td>
<td>pBCP330</td>
<td>0.6</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>\textit{ABCD}</td>
<td>pBCP330</td>
<td>0.6</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* The cells were grown overnight in minimal medium \textit{A} containing 0.4% gluconate in the absence or presence of 50 μM IPTG. Cells were harvested at an optical density at 600 nm of 1.2. \textit{PQQ} was measured enzymatically in the culture supernatant with apo-GCD and apo-EDH. Only the \textit{PQQ} concentrations obtained with apo-GCD are given. Growth on glucose minimal medium plates was judged after incubation at 37°C for 48 h; +, growth; −, no growth. Deletion or inactivation of a particular \textit{pqq} gene is indicated by a dash at the appropriate position.
mM, assuming that 1 mg of total cell protein is equivalent to an internal volume of 3.3 μl (40) and that all PQQ is localized in the cytoplasm. In the case of E. coli JA221/pBCP165 (pqqABC DEF), the PQQ concentration in the medium was 180 nM (the optical density at 600 nm was 1.2 when the cells and the supernatant were harvested). This means that more than 98% of the PQQ produced by the culture was present in the medium, assuming that an optical density at 600 nm of 1.0 corresponds to an internal volume of 600 μl per liter of culture (40).

In vitro complementation. Using cell extracts that contained all Pqq proteins except one, we investigated whether PQQ synthesis could be restored by adding a second extract containing the missing Pqq protein. Cell extracts lacking PqqA, D, or E did not contain or synthesize PQQ and could not be complemented in vitro by a cell extract containing the missing protein (Table 4).

In cell extracts lacking PqqF (pBCP186), the amount of PQQ was near the detection level (Table 4). Since pBCP186 contained a Tn10 in the middle of pqqF, possibly resulting in a truncated but partially active PqqF protein, pBCP499, which contained only 320 bp of pqqF (one-seventh of the gene), was constructed. Table 4 shows that small amounts of PQQ were present even when pqqF was almost completely deleted. In a cell extract lacking PqqF (pBCP186), PQQ synthesis could not be restored by the addition of a second extract containing PqqF (Table 4).

### Table 4. In vitro complementation

<table>
<thead>
<tr>
<th>Pqq proteins³</th>
<th>Plasmid(s)</th>
<th>Mean PQQ produced (pmol/mg of protein) ± SD</th>
<th>0 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>None</td>
<td>&lt;0.4</td>
<td>12.0 ± 3.0</td>
<td>11.5 ± 2.4</td>
</tr>
<tr>
<td>Extract lacking PqqA</td>
<td>pBCP325</td>
<td>ND± &lt;0.4</td>
<td>1.5 ± 0.5</td>
<td>5.5 ± 1.5</td>
</tr>
<tr>
<td>Extract lacking PqqB</td>
<td>pBCP328</td>
<td>ND± &lt;0.4</td>
<td>0.9 ± 0.2</td>
<td>6.5 ± 2.0</td>
</tr>
<tr>
<td>Extract lacking PqqC</td>
<td>pBCP329</td>
<td>ND± &lt;0.4</td>
<td>0.5 ± 0.1</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>Extract lacking PqqD</td>
<td>pBCP330</td>
<td>ND± &lt;0.4</td>
<td>0.9 ± 0.3</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Extract lacking PqqE</td>
<td>pBCP331</td>
<td>ND± &lt;0.4</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

* A combination of equal amounts of cell extracts, together containing all six Pqq proteins, and the single extracts were assayed. After 0 and 30 min, the reaction was stopped, and PQQ was measured with apo-GCD. For single extracts, only the value at 30 min is given if no PQQ was detectable. Values are given as mean ± standard deviation. When cell extracts were combined, the value given is based on the sum of the protein contents of both extracts.

³ Deletion or inactivation of a particular pqq gene is indicated by a dash at the appropriate position.

⁴ ND, not determined.
Table 4 also shows that extracts lacking PqqC could be complemented with extracts containing PqqC and that extracts lacking PqqB produced PQO. We will discuss this in more detail below.

In vitro complementation of a cell extract lacking PqqC. An extract containing all Pqq proteins except PqqC could be complemented in vitro by an extract containing PqqA, B, and C (pBCP337, Table 4). The production of PQO reached its maximum within 30 min (Fig. 3B). A plasmid that produced only PqqC, pBCP390, also restored PQO synthesis. The truncated pqqD gene from pBCP390 was not functional, since it could not complement pBCP338 [pqqABC(ΔD)EF] in vivo, the PQO concentration in the culture supernatant being less than 0.6 nM.

We determined the amount of PQO produced and its production rate in extracts containing all Pqq proteins except PqqC, supplemented with an extract containing PqqC as the only Pqq protein. The rate of PQO production increased with increasing amounts of PqqC-containing extract when the amounts of PqqA, B, D, E, and F were kept constant. The amount of PQO was produced (Fig. 4A). When the amount of cell extract containing all Pqq proteins except PqqC was increased while keeping the amount of PqqC-containing extract constant, the ratio of PQO production increased. The amount of PQO produced also increased with the amount of extract containing all proteins except PqqC (Fig. 4B). These results suggested that cells which lacked PqqC formed an intermediate in PQO biosynthesis which could be converted into PQO by a PqqC-containing cell extract. This was studied in more detail by measuring the amount of the intermediate and POQ at the start and at the plateau (after 30 min) of the reaction. Table 5 shows that during this in vitro complementation reaction, the intermediate was converted into POQ.

Excretion of intermediate by PqqC-lacking cells. Studies with E. coli cells harboring a plasmid that encoded all Pqq proteins except PqqC suggested that the defect in the pqqC gene resulted in excretion of an intermediate in PQO biosynthesis into the growth medium. The culture supernatant of JA221/pBCP329 [pqqAB(C46::Tn5sta1)DEF] incubated with an extract containing only PqqC produced POQ. The concentration of this intermediate in the supernatant was 2 to 8 nM. Production of the intermediate was also investigated in K. pneumoniae KA220, which lacks PqqC, transformed with plasmid pBCP329. The concentration of intermediate in the culture supernatant of K. pneumoniae KA220/pBCP329 was 25 to 60 nM. These concentrations of intermediate in the culture supernatant should be compared with the amount of POQ excreted by E. coli JA221/pBCP165 and K. pneumoniae NCTC 418/pBCP165, 180 and 540 nM, respectively.

PQO production by cell extracts lacking PqqB. Since strains lacking PqqB produced amounts of PQO barely above the detection level, it came as a surprise that an extract from E. coli carrying a plasmid lacking PqqB (pBCP328) produced POQ (6.5 pmol of PQO per mg of protein; Table 4). The maximal

Table 5. PQO and assayable PQO biosynthesis intermediate in E. coli JA221 and K. pneumoniae KA220 cell extracts containing various Pqq proteins*
amount of POQ produced was reached after 45 min. A small amount of POQ was detectable at the start of the experiment (Table 4 and Fig. 3C). Since in pBCP328 the Tn5lac1 element was inserted in the middle of the pqqB gene possibly producing a truncated but still active PqqB protein, pBCP324 [pqq4(B38::
Tn5lac1)]CDEF, in which a Tn5lac1 element was inserted 200 bp downstream from the start codon of pqqB, leaving only one-fifth of the functional gene intact, was constructed. Extracts made from cells harboring pBCP324 produced amounts of POQ comparable to those in an extract made from cells harboring pBCP328 (Table 4). Addition of an equal amount of a cell extract containing PqqA (pBCP176) to a pBCP328-derived extract resulted in a small stimulation of POQ production (Table 4) because the Tn10 insertion in the pqqC gene was not completely polar (data not shown). This increase in POQ production became evident when the amount of POQ was expressed as picomoles per milligram of protein of the PqqB-lacking extract rather than per milligram of protein of the sum of the protein contents of both extracts (as is done in Table 4). Calculated in this way, combination of a PqqB-lacking cell extract (pBCP328) with an extract containing PqqB (pBCP176) produced twice as much POQ (9 pmol of POQ per mg of protein in 30 min).

Cell extracts of K. pneumoniae KA220, which lacks PqqB, containing pBCP324 produced POQ in amounts comparable to the amounts produced in a cell extract from E. coli JA221/pBCP328, varying from 0.6 pmol of POQ per mg of protein at the start of the experiment to 13.5 pmol of POQ per mg of protein after 45 min. The supernatant of K. pneumoniae KA220/pBCP324 cells contained little POQ (concentration of POQ was less than 5 nM) compared with K. pneumoniae NCTC418 harboring pBCP165 (concentration of POQ was 540 nM).

Studies with E. coli and K. pneumoniae cell extracts lacking PqqB showed that they contained the same intermediate of POQ biosynthesis as cell extracts from PqqC-lacking cells. In vitro, POQ production in PqqB-lacking extracts was stopped at different times by the addition of HClO4, and the amount of POQ and POQ biosynthesis intermediate in the supernatant (after KClO4 removal) was determined. At the start of the experiment, this supernatant contained very little POQ (see above), but when PqqC was added, POQ was formed (Table 5). This meant that the same biosynthesis intermediate as in PqqC-lacking cells was present. During incubation of the PqqB-lacking extract at 37°C, this intermediate was converted into POQ (Table 5). In E. coli JA221 cell extracts lacking PqqA, PqqD, PqqE, or PqqF, this biosynthesis intermediate could not be detected.

Although the biosynthesis intermediate was detected in cell extracts made from K. pneumoniae cells lacking PqqB (KA220/pBCP324), it could hardly be detected in the growth medium. In the late exponential-early stationary phase, the concentration of intermediate in the supernatant of a K. pneumoniae KA220/pBCP324 cell culture was less than 3 nM, while the intermediate concentration in the cells was 4 μM. Under the same culture conditions, the intermediate concentration in PqqC-lacking K. pneumoniae KA222/pBCP329 cells was 5 μM, and its concentration in the culture supernatant was 25 to 60 nM.

**DISCUSSION**

The synthesis of POQ and its role as a cofactor in several dehydrogenases have been demonstrated in a number of bacteria (for a review, see reference 20). Although a number of pqq genes involved in POQ biosynthesis have been isolated from several bacteria, including A. calcoaceticus (15, 17), K. pneumoniae (25, 26), M. extorquens (28), and Erwinia herbicola (22), the function of these genes in POQ biosynthesis is unknown at present. The six K. pneumoniae pqq gene products show no similarity to other proteins in the database except for PqqF, which shows similarity with protease III from E. coli and some insulin-degrading enzymes (26). Interestingly, the three pqq operons that have been analyzed in some detail all contain a small gene (pqqA in K. pneumoniae) that could encode a polypeptide of 23 to 29 residues. All three polypeptides contain a glutamate and a tyrosine residue at conserved positions. Possible pathways for POQ biosynthesis starting with a tyrosine and a glutamate residue have been proposed (19, 44).

In this paper, we have examined the role of each of the six K. pneumoniae pqqABCDEF genes in POQ biosynthesis. Using an in vitro system, we have also detected an intermediate in POQ biosynthesis, and we have shown that the PqqC protein probably catalyzes the last step in POQ synthesis.

The role of each of the K. pneumoniae pqq genes in POQ biosynthesis in intact cells and in metabolism via a POQ-dependent pathway was studied in E. coli since E. coli can synthesize apo-glucose dehydrogenase, which oxidizes glucose to gluconate, but not its cofactor, POQ. Consequently, an E. coli pts mutant, which cannot metabolize glucose via the phosphotransferase system (the major pathway for glucose metabolism), grows slowly on glucose when POQ is added to the growth medium or when a plasmid which contains the pqq operon from K. pneumoniae is present (25). Our studies revealed that each of the six K. pneumoniae pqqABCDEF genes is required for growth on glucose via the glucose dehydrogenase-dependent pathway and for substantial POQ secretion into the medium. It is important to note that the pqqA gene complemented in trans and was required for POQ synthesis and excretion. This is in agreement with the hypothesis that the pqqA gene encodes the precursor polypeptide for POQ.

Our data show that almost no POQ was synthesized by K. pneumoniae harboring a plasmid containing the K. pneumoniae pqqABCDEF genes under anaerobic growth conditions, although the expression of several pqq-lacZ operon fusions was not impaired, suggesting that the Pqq enzymes were synthesized under anaerobic conditions. Most likely, a hydroxylase, requiring molecular oxygen, is involved in the biosynthesis of POQ for the formation of the quinone groups (19, 44). We cannot presently exclude the possibility, however, that one or more enzymes involved in POQ biosynthesis are inactive in the absence of oxygen.

Using pqq-lacZ operon fusions localized on the K. pneumoniae chromosome, we also studied the expression level of the K. pneumoniae pqq genes. The β-galactosidase activity decreased about sevenfold within the pqq operon, fusions located at the end of the operon having the lowest activity. These results confirm our earlier conclusion that besides the pqqA promoter, which was mapped by primer extension analysis to lie upstream of pqq4 (26), no other strong internal promoters were present. The β-galactosidase activity of the different pqq-lacZ operon fusions indicated that the transcription of the pqq genes was low, the highest activity being that of the pqq-lacZ fusion located between pqq4 and pqqB. The value was 5- to 10-fold lower than that of lacZ fusions to K. pneumoniae genes encoding metabolic enzymes, such as the sor (sorbose) and gut (D-glucitol) genes (46).

To study in more detail the role of the various Pqq proteins in POQ biosynthesis, we have developed an in vitro system for POQ synthesis by combining extracts containing all but one of the Pqq proteins with an extract containing the missing protein. An E. coli cell extract made from cells in which all six Pqq
proteins were present contained 12.0 ± 3.0 pmol of POQ per mg of protein. Extracts lacking the PqqA, PqqC, PqqD, PqqE, or PqqF protein contained no POQ or amounts below the detection level (except maybe in the case of PqqF; see below). A certain amount of POQ was detected in extracts of PqqB-deficient cells, however.

In vitro complementation could be clearly demonstrated in the case of PqqC. POQ was produced when a cell extract containing all Pqq proteins except PqqC was combined with a cell extract that contained PqqC. The separate extracts produced no POQ. These results strongly suggest that an intermediate in POQ synthesis had accumulated in cells lacking PqqC. The putative intermediate was also detected in the culture medium of E. coli and K. pneumoniae cells lacking PqqC and could be converted into POQ with a cell extract containing only PqqC. This result suggested that PqqC is the last enzyme of the pathway and that the intermediate is a POQ-like molecule rather than a polypeptide resembling PqqA. However, it cannot be completely excluded that present other enzymes, not encoded by the known pqq genes but present in E. coli and K. pneumoniae, are required for the conversion of the putative intermediate into POQ. At present, we are purifying and characterizing the detected intermediate.

In all other cases, reconstitution of POQ biosynthesis by combining the various extracts was not successful. It is important to note that in all cases, in vivo complementation was observed with the same plasmids from which the Pqq proteins in these cell extracts were derived. Possibly, complexes between two or more Pqq proteins have to be formed for proper functioning, a process that may occur only during the synthesis of these proteins in the intact cell. Alternatively, the concentration of one or more Pqq proteins may be too low in the extracts compared with their concentration in an intact cell. Finally, some intermediates in POQ biosynthesis, when accumulated in the various pqq mutants, as well as one or more of the Pqq proteins might be unstable under the conditions used to prepare and incubate the extracts.

Our studies on the role of the PqqB protein have yielded unexpected results. E. coli cells containing (on a plasmid) all pqq genes except pqqB excreted little if any POQ into the growth medium (Table 3). Similarly, an E. coli mutant unable to grow on glucose via the phosphotransferase pathway could not metabolize glucose via the POQ-dependent glucose dehydrogenase pathway if the pqqB gene was lacking. These results point to an essential role for PqqB in POQ biosynthesis. To our surprise, however, a cell extract, containing all Pqq proteins except PqqB could produce POQ in vitro in a time-dependent manner. It should be noted, however, that the rate of POQ production in a PqqB-lacking cell extract is relatively low compared with that catalyzed by the PqqC-containing extract (compare Fig. 3B and C). PqqB homologs have been found in A. calcoaceticus (PqqV) and M. extorquens (PqqG), but conflicting results about their role have been reported. The A. calcoaceticus PqqV protein was reported not to be necessary for growth via a POQ-requiring pathway (15, 17), whereas in the case of M. extorquens AM1, it was concluded that the PqqG protein was required for POQ biosynthesis (28), similar to PqqB in E. coli.

These conflicting results may be explained by our observations with extracts made from cells lacking the pqqB gene. These cells contained the same intermediate which we have detected in PqqC-deficient cells but could not convert it into POQ, although functional PqqC was present. Furthermore, the intermediate could hardly be detected in the growth medium of these PqqB-deficient cells, although the intracellular concentration was comparable to that of PqqC-lacking cells. Possibly, PqqB is involved in the transport of POQ across the cytoplasmic membrane into the periplasm. Since there is no evidence that PqqB contains hydrophobic stretches, it is unlikely that PqqB itself can transport POQ across the membrane. However, PqqB could modify an existing transport system so that secretion of POQ becomes possible. Lack of PqqB could cause accumulation of POQ in the cytoplasm and subsequent inhibition of PqqC activity, resulting in an increased concentration of the intermediate in the cytoplasm. This hypothesis would also explain why in a cell extract made from PqqB-deficient cells, in which the cell contents (e.g., POQ) become diluted, PqqC would become active. The PqqB-dependent transport system might also recognize the intermediate. As a consequence, the intermediate would be secreted by cells lacking PqqC but containing PqqB. This is in agreement with our findings.

We have shown that E. coli cell extracts made from cells containing all Pqq proteins except the protease III-like PqqF protein contained a small amount of POQ, just above the detection limit. These cells also produced some POQ in the culture medium, although the final concentration was at least 100-fold lower than that produced by cells harboring all six Pqq proteins, suggesting that small but measurable amounts of POQ might be produced in the absence of PqqF. In previous studies, we reported that the PqqF protein is necessary for the substantial conversion of glucose into gluconate via POQ-dependent glucose dehydrogenase, which is required for growth of a K. pneumoniae ptsI mutant on glucose via this pathway (26). We have recently observed, however, that a plasmid containing the K. pneumoniae pqqABCD genes but lacking the pqqF gene restored growth on glucose of ptsI derivatives of E. coli JM109 and HB101 but not of some other E. coli K-12 strains (3a). Possibly, protease III or other protease III-like enzymes can, to a limited extent, substitute for PqqF in POQ biosynthesis, i.e., produce small amounts of POQ. This might explain the observation by Goosen and coworkers (15) that a plasmid containing the five known A. calcoaceticus pqq genes, which showed similarity to pqqA, B, C, D, and E of K. pneumoniae, restored growth of an E. coli ptsI mutant on glucose minimal medium. It is important to note, however, that not all E. coli strains supplied with all pqq genes except pqqF on a plasmid can synthesize POQ in amounts sufficient to support growth on glucose via glucose dehydrogenase. Thus, this proposal requires that the enzyme substituting for PqqF be present in some E. coli strains at higher levels than in others. We are presently in the process of identifying this PqqF-substituting enzyme.

We have mentioned previously the hypothesis that the small PqqA polypeptide might be a precursor in POQ biosynthesis. This would require synthesis of PqqA in stoichiometric amounts rather than catalytic amounts compared with the other Pqq proteins. Using a plasmid with the pqqA gene cloned behind an inducible T7 promoter, we could demonstrate that a polypeptide with a mobility expected for PqqA is indeed synthesized. The level of expression of various pqq-lacZ protein fusions demonstrated clearly that expression of the pqqA gene was much higher (at least 20-fold) than the expression of other pqq genes like pqqC and pqqE. The drop in expression of the genes downstream of pqqA might be caused by transcriptional termination within the operon. This is supported by analysis of the mRNA sequence between pqqA and pqqB, which revealed a hairpin structure between nucleotides 1034 and 1053 of the published sequence of the pqq operon (26). A hairpin was also found downstream of pqqIV (15) and pqqD (30), the genes corresponding to pqqA in A. calcoaceticus and M. extorquens AM1, respectively. In M. extorquens AM1, the transcript en-
cording PqqD was more abundant than the transcripts encoding PqqB and PqqQ (the homolog of K. pneumoniae PqqB) (30). In conclusion, we think it is likely that the mRNA which terminates at the hairpin codes for a PqqA polypeptide. Together with the relatively high expression of the pqqJ gene compared with that of the other pqq genes, this supports the hypothesis that PqqA is the precursor for PQQ synthesis.

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