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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 proteins 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.

Pyrroloquinoline quinone (PQQ) is a cofactor of several bacterial dehydrogenases and transfers redox 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 *Methylobacterium 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 ^{13}C 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 *Klebsiella 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 *C*, 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* PqqIII (22). 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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TABLE 1. Bacterial strains, phages, and plasmids used[a]

Strain, phage, or plasmid	Relevant genotype or properties	Source or reference
E. coli strains		
ED8654	*supE supF metB ton*$^+$ *hsdR*	5
W3350	*sup*° *lac gal*	7
JA221	*thr leu thi* Δ(*trpE*)*5 lac gal xyl mtl phx hsdR recA supE*	10
ZSC112	*ptsM ptsG glk thi*	11
BL21(DE3)	F$^-$ *ompT* $r_B{}^-$ $m_B{}^-$ λDE3 (carries gene for T7 RNA polymerase under *lacUV5* control)	41
MC1060	Δ(*lacIYZA*) *galU galK rpsL hsdR*	8
K. pneumoniae strains		
NCTC418	Wild-type prototroph	32
KA56	*ptsI103*, P1 sensitive	25
KA196	*lacZ100*::Tn*10* minitet	This work
KA197	*lacZ100*::Tn*10* minitet *pqq-18*::Tn*5lacZ*	This work
KA202	*lacZ100*::Tn*10* minitet *pqqE22*::Tn*5lacZ*	This work
KA204	*lacZ100*::Tn*10* minitet *pqqB24*::Tn*5lacZ*	This work
KA220	*ptsI103 pqqB38*::Tn*5tac1*	This work
KA222	*ptsI103 pqqC40*::Tn*5tac1*	This work
Phages		
λES1	λ*sbhI* λ1° (*srl*λ1-2) Δ(*att int red gam*) Δ*pqq cI*857 *S*am7	24
λb20	Contains Tn*5* with promoterless *lacZ* gene in IS*50*L	37
λNK1098	Contains Tn*10* minitet	45
λ::Tn*5tac1*	Tn*5tac1*	9
Plasmids		
pBCP138	*pqqABCD* and >90% of *E*; Cm^r	25
pBCP141	*pqqAB*(*C2*::Tn*10*Km)*D* and >90% of *E*; Cm^r	26
pBCP162	*pqqABCDEF*; Ap^r	25
pBCP164	*pqqABCDEF*; Ap^r Tc^r	25
pBCP165	*pqqABCDEF*; Cm^r	25
pBCP168	*pqqABCDEF*; Cm^r	This work
pBCP176	*pqqAB*(*C2*::Tn*10*)*DEF*; Ap^r Km^r Tc^r	This work
pBCP186	*pqqABCDE*(*F17*::Tn*10*); Ap^r Km^r Tc^r	25, 26
pBCP272	*pqqA*(*B38*::Tn*5tac1*)*CD* and >90% of *E*; Cm^r Km^r	This work
pBCP274	*pqqAB*(*C40*::Tn*5tac1*)*D* and >90% of *E*; Cm^r Km^r	This work
pBCP324	*pqqA*(*B38*::Tn*5tac1*)*CDEF*; Ap^r Km^r Tc^r	This work
pBCP325	*pqqBCDEF*; Cm^r	26
pBCP328	*pqqA*(*B45*::Tn*5tac1*)*CDEF*; Cm^r Km^r	This work
pBCP329	*pqqAB*(*C46*::Tn*5tac1*)*DEF*; Cm^r Km^r	This work
pBCP330	*pqqABCD*(*E47*::Tn*5tac1*)*F*; Cm^r Km^r	This work
pBCP335	*pqqA*; Ap^r	This work
pBCP337	*pqqABC*; Ap^r	This work
pBCP338	*pqqABC*[Δ(*D*)*48*]*EF*; Cm^r	This work
pBCP341	*pqqABCD*; Ap^r	This work
pBCP352	*pqqABCDEF* behind λ p_L cloned at start codon of *pqqA*; Ap^r	This work
pBCP361	ϕ(*pqqA′-lacZ*)*49* (Hyb); Ap^r	This work
pBCP362	*pqqAB*ϕ(*C′-lacZ*)*50* (Hyb); Ap^r	This work
pBCP363	*pqqABCD*ϕ(*E′-lacZ*)*51* (Hyb); Ap^r	This work
pBCP364	*pqqAB* behind T7 promoter cloned at start codon of *pqqA*; Ap^r	This work
pBCP390	*pqqC*; Ap^r	This work
pBCP499	*pqqABCDE*; Ap^r	This work
pJF118HE	Contains *tac* promoter, rbs, mcs, and *lacI*^{q}; Ap^r	1, 6, 14
pJF119HE	Same as pJF118HE, but larger mcs	1, 6, 14
pAMH62	Encodes *lamB*; Ap^r	18
pET-3b	Contains T7 promoter, rbs, *lacI*, mcs with *Nde*I site; Ap^r	41
pNM480	Vector for constructing *lacZ* protein fusions; Ap^r	38
pNM481	Same as pNM480, but other reading frame	38
pRE1	Contains λp_L, rbs, mcs with *Nde*I site; Ap^r	31
pBR322	Ap^r Tc^r	Cloning vector

[a] Abbreviations: P1, phage P1; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Hyb, hybrid; mcs, multiple cloning site; rbs, ribosome-binding site.

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

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MATERIALS AND METHODS

Bacterial strains, phages, plasmids, and growth media. The bacterial strains, phages, and plasmids 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-

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 *tac* 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 assaying phage and phage DNA were those described by Arber et al. (3). Transformation, digestion, and ligation were performed by standard procedures (34). Restriction and modification enzymes and buffers were obtained from Pharmacia, Biozym, 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 Tn*10* insertion in the *K. pneumoniae lacZ* gene, *K. pneumoniae* NCTC418 was made sensitive to bacteriophage λ by the introduction of plasmid pAMH62 and then infected with λNK1098, as described by Way et al. (45). White colonies were selected on Luria agar plates containing tetracycline, 5-bromo-4-chloro-3-indoyl-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 *pqqB* or *pqqC* was constructed by transferring the *pqqB38*::Tn*5tac1* allele (from pBCP272) or the *pqqC40*::Tn*5tac1* allele (from pBCP274) to the chromosome of *K. pneumoniae* KA56 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 incubating *E. coli* W3350/pBCP138 with λb20 (containing a Tn*5* with a promoterless *lacZ* gene in the left-end inverted repeat of Tn*5* [IS*50*L] [37]) for 30 min at 37°C and isolating kanamycin- and chloramphenicol-resistant colonies on Luria agar plates. Plasmid DNA from the pooled mutants was transformed into *E. coli* MC1060, and blue transformants were selected on Luria agar plates containing kanamycin, chloramphenicol, and X-Gal. The location of the Tn*5lacZ* insertion was determined by restriction analysis. The fusions were transferred to the chromosome of KA196 with λES1 as described elsewhere (24), yielding KA197 (*pqq-18*::*lacZ*; insertion between *pqqA* and *pqqB*), KA204 (*pqqB24*::*lacZ*), and KA202 (*pqqE22*::*lacZ*; insertion in the middle of *pqqE*) (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 *pqqB* start codon) were determined by sequencing the fusion points.

Construction of plasmids. (i) Plasmids with an incomplete set of *pqq* genes. Nonpolar insertions of the Tn*5tac1* element (9) in the *pqqB* and *pqqC* genes of pBCP138 were isolated by infection of *E. coli* W3350/pBCP138 with λ::Tn*5tac1*. The insertion point of the Tn*5tac1* element of the resulting plasmids, pBCP272 (*pqqB38*::Tn*5tac1*, insertion approximately 200 bp downstream of the start codon) and pBCP274 (*pqqC40*::Tn*5tac1*, insertion approximately 700 bp downstream of the start codon), was determined by restriction enzyme analysis.

Plasmid pBCP168, containing the complete *K. pneumoniae pqq* operon, was constructed by ligating the 2.6-kb *Xho*I-*Hin*dII fragment of pBCP162 (25) (this fragment contained *pqqF* and part of *pqqE*) into pBCP138 digested with *Xho*I and *Hin*dIII. Tn*5tac1* insertions were isolated in the *pqqB*, *pqqC*, and *pqqE* genes of pBCP168 by using *E. coli* W3350/pBCP168. The insertion points of the Tn*5tac1* elements in *pqqB* (pBCP328, approximately 500 bp downstream of the start codon), *pqqC* (pBCP329, approximately 600 bp downstream of the start codon), and *pqqE* (pBCP330, approximately 100 bp downstream of the start codon) were determined by restriction enzyme analysis (Fig. 1C).

To construct a plasmid in which Tn*5tac1* was inserted closer to the *pqqB* start codon than in pBCP328, phage λES1 was grown on *E. coli* ED8654/pBCP272, and the lysate was used to infect *E. coli* W3350/pBCP164. In the resulting plasmid, pBCP324 (Fig. 1C), correct integration of the Tn*5tac1* element was confirmed by restriction enzyme analysis.

A plasmid with a defective *pqqD* gene was constructed by deleting the internal 63-bp *Aat*II fragment of *pqqD* of pBCP168. This deletion caused a nonpolar mutation in the *pqqD* gene, and the resulting plasmid was designated pBCP338 (Fig. 1C).

A plasmid containing all *pqq* genes except *pqqF* was constructed by digestion of pBCP168 with *Acc*I and filling in the *Acc*I site with Klenow polymerase. After a second digestion with *Nhe*I, the resulting 3.7-kb *Nhe*I blunt-ended fragment, which contained the *pqq* promoter (up to 220 bp upstream of the *pqqA* start codon), the *pqqABCDE* genes, and 320 bp of *pqqF* (i.e., one-seventh of the gene), was ligated into the vector pJF119HE digested with *Xba*I and *Sma*I, resulting in pBCP499 (Fig. 1C).

A plasmid containing only the *pqqA* gene was constructed by ligation of the 1.0-kb *Pst*I-*Eco*RV fragment of pBCP165 (25), containing *pqqA* and the *pqq* promoter, into the vector pJF118HE digested with *Pst*I and *Sma*I, resulting in pBCP335 (Fig. 1C).

Plasmid pBCP390 (Fig. 1C), containing only the *pqqC* gene, was constructed by digesting pBCP165 with *Tth111*I and filling in the *Tth111*I site with Klenow polymerase. After a second digestion with *Sph*I, the resulting 0.97-kb *Sph*I blunt-ended fragment, which contained *pqqC* and 213 bp of *pqqD*, was ligated into the vector pJF119HE digested with *Sph*I and *Sma*I.

To construct pBCP176 (Fig. 1C), containing only functional *pqqA* and *pqqB* genes, the *pqqC2*::Tn*10Km* allele from pBCP141 (26) was transferred to pBCP164 by using λES1 as described for the construction of pBCP324 in this section.

A plasmid containing only the functional *pqqA*, *B*, and *C* genes was constructed by deleting the internal *Hin*dIII fragment of a *pqqD*::Tn*5lacZ* insertion (containing the kanamycin resistance and part of the IS*50*R sequence [23]). After digestion with *Eco*RI and *Sal*I, the 6.0-kb fragment (containing the complete *pqqA*, *B*, and *C* genes and part of *pqqD*::Tn*5lacZ*) was ligated into pBR322 digested with *Eco*RI and *Sal*I, resulting in pBCP337 (Fig. 1C).

To construct a plasmid containing *pqqABCD*, the internal *Hin*dIII fragment of a Tn*5lacZ* insertion in *pqqE* was deleted. Ligation of the 6.5-kb *Eco*RI-*Sal*I fragment of this plasmid (containing the *pqqA*, *B*, *C*, and *D* genes and part of *pqqE*::Tn*5lacZ*) into pBR322 digested with *Eco*RI and *Sal*I resulted in pBCP341 (Fig. 1C).

(ii) Plasmids with *pqq-lacZ* protein fusions. Several *pqq-lacZ* protein fusions (Fig. 1B) were constructed with the help of pNM480 and pNM481 (38).

To obtain a *pqqA-lacZ* protein fusion, a 0.5-kb DNA fragment containing part of ORFX, the *pqq* promoter, and 51 nucleotides of *pqqA* (encoding 17 amino acids) was amplified by PCR. The primer downstream of the *pqqA* start codon contained a *Bam*HI site, and the primer upstream of the *pqqA* start codon (in ORFX) contained a *Sal*I site. This PCR fragment was digested with *Sal*I, and the *Sal*I site was filled in with Klenow polymerase. After a second digestion with *Bam*HI, the resulting 0.5-kb fragment was ligated into pNM481 which had been digested with *Sma*I and *Bam*HI, resulting in pBCP361.

A *pqqC-lacZ* protein fusion was constructed by ligating the 2.2-kb *Bam*HI fragment of pBCP138, containing the *pqq* promoter, the *pqqA* and *pqqB* genes, and part of the *pqqC* gene, into pNM481 digested with *Bam*HI. This resulted in pBCP362.

To construct a plasmid with a *lacZ* fusion in *pqqE*, pBCP168 was digested with *Sal*I, and the *Sal*I site was filled in with Klenow polymerase. After a second digestion with *Bgl*II, the resulting 3.2-kb blunt-ended *Bgl*II fragment (containing the *pqq* promoter, the *pqqA*, *B*, *C*, and *D* genes, and part of the *pqqE* gene) was ligated into pNM480 digested with *Sma*I and *Bam*HI, resulting in pBCP363.

(iii) Plasmid with *pqqA* behind the T7 promoter. To construct a plasmid with the *pqqA* gene under control of the T7 promoter, an *Nde*I site was created at the *pqqA* start codon by site-directed mutagenesis (21). The mutated fragment was sequenced to confirm that no additional mutations had occurred and cloned (together with the other *pqq* genes) in pRE1. The resulting plasmid, pBCP352, in which all six *pqq* genes were under control of the heat-inducible λ p_L promoter, produced PQQ upon heat induction (data not shown). The 1.3-kb *Nde*I-*Bam*HI fragment of pBCP352 (containing *pqqAB* and part of *pqqC*) was ligated into pET-3b (41) digested with *Nde*I and *Bam*HI. In the resulting plasmid, pBCP364, *pqqA* 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 gluconate and centrifuged at 10,000 × *g* for 10 min. The pellet was washed twice with 0.5 volume of 0.9% NaCl and resuspended in 1/100th of the original culture volume of 25 mM potassium phosphate buffer, pH 7, containing 0.5 mM EDTA. The cells were ruptured by passage through an Aminco French pressure cell at 1,000 kg/cm^2, and the cell extracts were centrifuged for 10 min at 13,000 rpm in an Eppendorf centrifuge to remove cell debris. The supernatant was divided into Eppendorf vials, frozen in liquid N_2, and stored at −80°C until use. Portions of cell extract were thawed on ice directly before use and used only once. For cell cultures harboring plasmids with a *tac* promoter in front of *pqq* genes (except pBCP335), IPTG to a final concentration of 50 μM was added during growth. Plasmid pBCP335 contained the *tac* promoter upstream of the *pqq* promoter and the *pqqA* gene. Since the *pqq* promoter alone was sufficient to direct expression of *pqqA*, no IPTG was added.

Preparation of culture supernatant for measurement of PQQ and intermediate of PQQ biosynthesis. To measure PQQ production and excretion into the medium, *E. coli* JA221 containing one or more plasmid-borne *pqq* genes or *K. pneumoniae* NCTC418/pBCP165 was grown overnight at 37°C in minimal medium A containing gluconate, in the presence or absence of 50 μM IPTG. The cells were centrifuged at 13,000 rpm in an Eppendorf centrifuge for 5 min, and the culture supernatant was used for the measurement of PQQ.

To measure the production and excretion of the PQQ biosynthesis intermediate, *E. coli* JA221/pBCP329 or *K. pneumoniae* KA222/pBCP329 cells were grown in minimal medium A containing gluconate in the presence of 50 μM IPTG at 37°C until the late exponential-early stationary phase (final optical density at 600 nm was 0.8 to 1.1). The cells were centrifuged at 13,000 rpm in an Eppendorf centrifuge for 5 min. The supernatant was immediately stored on ice and assayed within 1 h for the presence of the intermediate.

In vitro synthesis of PQQ. To measure PQQ synthesis in vitro, 50 μl of cell extract (one extract or a combination of two extracts; 0.1 to 0.6 mg of protein) was added to 150 μl of 100 mM 1,4-piperazinediethane sulfonic acid (PIPES) in a 2-ml Eppendorf vial and incubated at 37°C with shaking. At various times, the reaction was stopped by adding $HClO_4$ to a final concentration of 5% (vol/vol). After incubation on ice for 20 to 60 min followed by neutralization with 5 M KOH to pH 7, the reaction mixture was incubated on ice for another 10 min and centrifuged for 5 min at 13,000 rpm in an Eppendorf centrifuge to remove the $KClO_4$ precipitate. PQQ in the supernatant was determined with apo-glucose

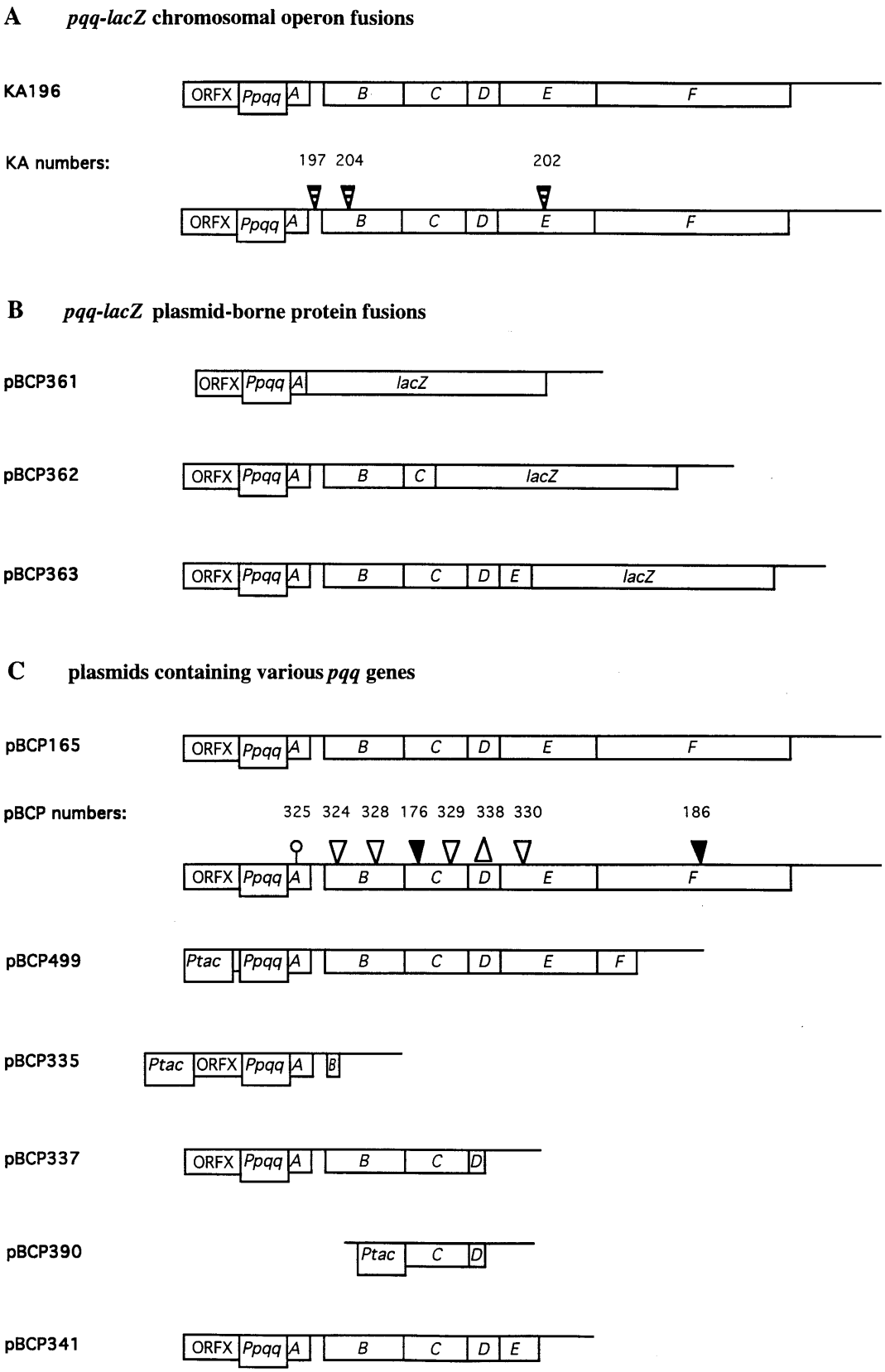

FIG. 1. Schematic representation of *pqq-lacZ* fusions and *pqq* plasmids. Details of the construction of strains and plasmids are given in Materials and Methods. The *pqq* genes, in particular *pqqA*, are not drawn completely to scale. *Ptac*, *tac* promoter; *Ppqq*, *pqq* promoter. Symbols: ⍒, Tn*5lacZ* element; ⚲, *Eco*RI linker; ▽, Tn*5tac1* element; △, deletion; ▼, Tn*10Km* 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. Olsthoorn, 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 $CaCl_2$ 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 $CaCl_2$, 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 A_{600} 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 μM apo-EDH and 5 mM $CaCl_2$. 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 1-butanol, 2.4 mM $CaCl_2$, and 1.5 mM Würsters Blue. The decrease in A_{612} was measured and corrected for reduction of Würsters Blue in the absence of EDH.

Assay for intermediate in PQQ biosynthesis. To determine the amount of intermediate in PQQ biosynthesis, 50 μl of sample (culture supernatant or supernatant from cell extract[s] after $HClO_4$/KOH treatment; see above) was added to a PqqC-containing extract (0.04 to 0.065 mg of protein) in 120 μl of 0.1 M Tris-HCl (pH 7.5) containing 0.02 μM apo-GCD and 3 mM $CaCl_2$. 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 the possible PQQ already present, the sample was also assayed for PQQ with apo-GCD.

β-Galactosidase assay. 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 is 1).

Synthesis of PqqA. *E. coli* BL21(DE3), carrying on its chromosome the gene for T7 RNA polymerase under *lacUV5* control, was transformed with pBCP364, containing *pqqA* behind the T7 promoter. The transformed cells were grown in LB at 37°C to an optical density at 600 nm of 0.8. After induction of the T7 polymerase gene by the addition of IPTG to a final concentration of 400 μM, the cells were grown for an additional hour, harvested by centrifugation, and subjected to tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) (35).

Protein determination. The amount of protein was determined with the bicinchoninic acid (Sigma) method (39). The assay 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 N_2 for 30 min. Because 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) preculture.

TABLE 2. Expression of *pqq*-Tn*5lacZ* operon fusions on the chromosome of *K. pneumoniae*[a]

Strain	Fusion	β-Galactosidase activity (nmol of ONPG hydrolyzed/min/ml of cell culture)		
		Aerobic (LB)	Aerobic (MM)	Anaerobic (MM)
KA196	None	1.2	1.2	0.9
KA197	Between *pqqA* and *pqqB*	10	12.5	10.2
KA204	*pqqB-lacZ*	4.8	7.0	6.1
KA202	*pqqE-lacZ*	1.5	ND[b]	ND
NCTC418[c]	None; wild-type *lacZ*	146	ND	ND

[a] Cells were grown in batch culture in LB or in minimal medium A containing 0.4% gluconate (MM) and harvested in the exponential phase.
[b] ND, not determined.
[c] After induction with 1 mM IPTG.

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-18*::Tn*5lacZ*) and KA204 (*pqqB24*::Tn*5lacZ*) (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

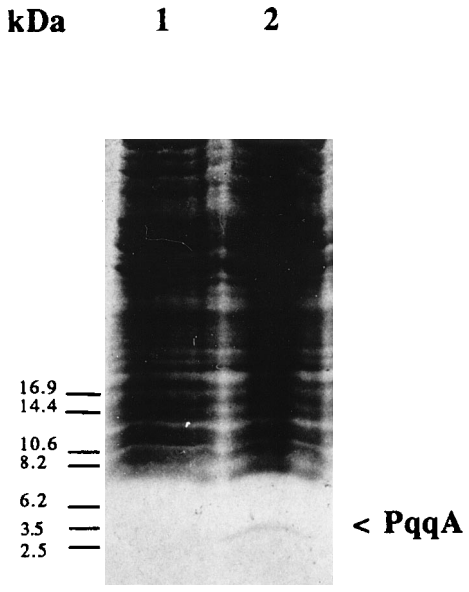

FIG. 2. Synthesis of the *pqqA* gene product. *E. coli* BL21(DE3) cells carrying a plasmid with *pqqA* cloned behind the T7 promoter (pBCP364) were grown to an optical density at 600 nm of 0.8 in the presence or absence of IPTG. Total cell protein was analyzed by tricine-SDS-PAGE as described in Materials 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.

TABLE 3. PQQ in the culture supernatant of *E. coli* JA221, and growth on glucose minimal medium of *E. coli* ZSC112 containing various *pqq* plasmids[a]

pqq genes	Plasmid(s)	IPTG	PQQ concn (nM)	Growth on glucose
Controls				
None	None	−	<0.6	−
ABCDEF	pBCP165	−	180	+
Complementation with *pqqA*				
—BCDEF + A	pBCP325 + pBCP335	−	66	
—BCDEF	pBCP325	−	<0.6	−
A	pBCP335	−	<0.6	
Complementation with *pqqB*				
A—CDEF + AB	pBCP328 + pBCP176	+	50	
A—CDEF	pBCP328	+	0.6	−
A—CDEF	pBCP324	+	1	
AB	pBCP176	−	<0.6	
Complementation with *pqqC*				
AB—DEF + ABC	pBCP329 + pBCP337	+	144	
AB—DEF + C	pBCP329 + pBCP390	+	30	
AB—DEF	pBCP329	+	<0.6	−
ABC	pBCP337	−	<0.6	
C	pBCP390	+	<0.6	
Complementation with *pqqD*				
ABC—EF + ABCD	pBCP338 + pBCP341	−	48	
ABC—EF	pBCP338	−	<0.6	−
ABCD	pBCP341	−	<0.6	
Complementation with *pqqE* or *pqqF*				
ABCD—F + ABCDE	pBCP330 + pBCP186	+	96	
ABCDE + —BCDEF	pBCP499 + pBCP325	+	11	
ABCD—F	pBCP330	+	<0.6	−
ABCDE	pBCP186	−	0.6	−
ABCDE	pBCP499	+	0.7	

[a] The cells were grown overnight in minimal medium 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. PQQ was measured enzymatically in the culture supernatant with apo-GCD and apo-EDH. Only the 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 *pqq* gene is indicated by a dash at the appropriate position.

expected that *pqqA* would encode a polypeptide which is produced in higher amounts than the other Pqq proteins. To compare the expression of the different *pqq* genes, *lacZ* fusions were constructed with *pqqA* (pBCP361), *pqqC* (pBCP362), and *pqqE* (pBCP363) (see Fig. 1B). The activity of the fusion proteins was measured in *E. coli* MC1060. The *lacZ* fusion in *pqqA* resulted in a 20-fold-higher β-galactosidase activity (500 nmol of ONPG/min/ml of culture) than the *lacZ* fusions in *pqqC* and *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 *pqq* genes (*pqqABCDEF*) were necessary for 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 *pqq* genes, were transformed together into the *E. coli recA* strain JA221. As a control, each of the plasmids was transformed separately. The cells containing the various *pqq* plasmids were grown overnight in minimal medium containing gluconate, and 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 *pqq* genes was present resulted in PQQ synthesis and excretion. No PQQ was detected in supernatants from cell cultures harboring only a single plasmid which lacked either *pqqA*, *C*, *D*, or *E*. In supernatants of cell cultures harboring plasmids lacking *pqqB* (pBCP324 and pBCP328) or *pqqF* (pBCP186 and pBCP499), small amounts of PQQ, only slightly above the detection level, were measured (Table 3).

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

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

TABLE 4. In vitro complementation[a]

Pqq proteins[b]	Plasmid(s)	Mean PQQ produced (pmol/mg of protein) ± SD	
		0 min	30 min
Controls			
None	None	<0.4	<0.4
ABCDEF	pBCP165	12.0 ± 3.0	11.5 ± 2.4
Extract lacking PqqA			
—BCDEF	pBCP325	ND[c]	<0.4
A	pBCP335	ND	<0.4
—BCDEF + A	pBCP325 + pBCP335	<0.4	<0.4
Extracts lacking PqqB			
A—CDEF	pBCP324	1.5 ± 0.5	5.5 ± 1.5
A—CDEF	pBCP328	0.9 ± 0.2	6.5 ± 2.0
AB	pBCP176	ND	<0.4
A—CDEF + AB	pBCP328 + pBCP176	0.5 ± 0.1	4.5 ± 1.0
Extract lacking PqqC			
AB—DEF	pBCP329	ND	<0.4
ABC	pBCP337	ND	<0.4
C	pBCP390	ND	<0.4
AB—DEF + ABC	pBCP329 + pBCP337	<0.4	6.5 ± 1.5
AB—DEF + C	pBCP329 + pBCP390	<0.4	9.5 ± 1.5
Extract lacking PqqD			
ABC—EF	pBCP338	ND	<0.4
ABCD	pBCP341	ND	<0.4
ABC—EF + ABCD	pBCP338 + pBCP341	<0.4	<0.4
Extract lacking PqqE or PqqF			
ABCD—F	pBCP330	ND	<0.4
ABCDE	pBCP186	0.9 ± 0.3	1.0 ± 0.4
ABCDE	pBCP499	0.5 ± 0.1	0.5 ± 0.1
ABCD—F + ABCDE	pBCP330 + pBCP186	0.7 ± 0.3	0.5 ± 0.1

[a] 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 PQO was measured with apo-GCD. For single extracts, only the value at 30 min is given if no PQO 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.

[b] Deletion or inactivation of a particular *pqq* gene is indicated by a dash at the appropriate position.

[c] ND, not determined.

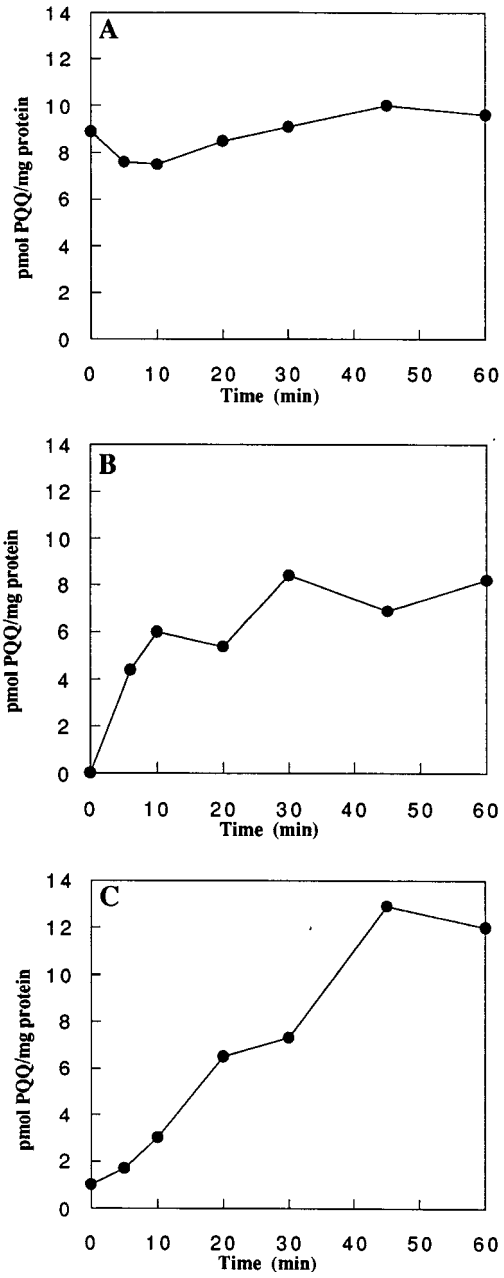

FIG. 3. In vitro PQO synthesis. In vitro PQO synthesis was measured in cell extracts (one extract or a combination of two extracts) from *E. coli* JA221 cells harboring *pqq* genes on a plasmid. The extract was incubated at 37°C, and the reaction was stopped at various times, as described in Materials and Methods. The amount of PQO was determined with apo-GCD. When cell extracts were combined, the values were based on the sum of the protein contents. (A) Cell extract (0.25 mg of protein) containing PqqA, B, C, D, E, and F (pBCP165). (B) Combination of a cell extract (0.22 mg of protein) containing PqqA, B, D, E, and F (pBCP329) with an extract (0.22 mg of protein) containing PqqA, B, and C (pBCP337). (C) Cell extract (0.20 mg of protein) containing PqqA, C, D, E, and F (pBCP328).

μM, 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 Tn*10* 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).

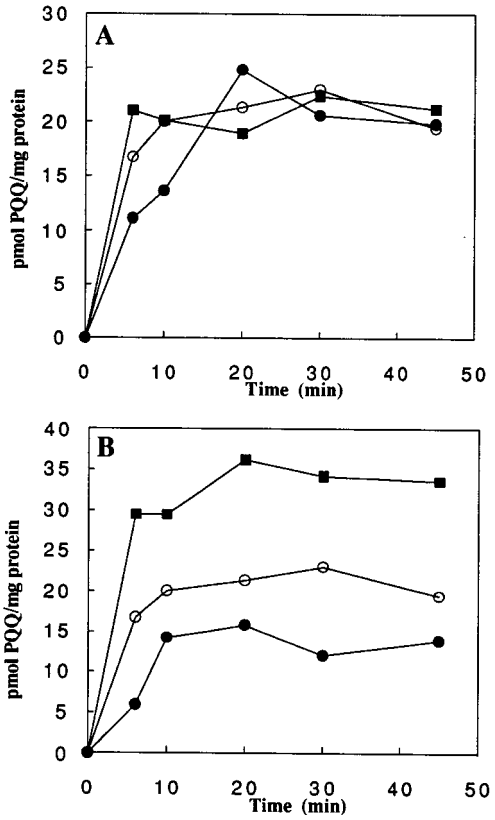

FIG. 4. In vitro PQQ synthesis. A constant amount of *E. coli* JA221/pBCP329 cell extract was combined with various amounts of *E. coli* JA221/pBCP390 cell extract, and vice versa. After incubation at 37°C, the reaction was stopped at various times, as described in Materials and Methods. The amount of PQQ was determined with apo-GCD. (A) A constant amount of cell extract (0.1 mg of protein) containing PqqA, B, D, E, and F (pBCP329) was combined with various amounts of cell extract containing PqqC (pBCP390); ●, 0.05 mg; ○, 0.1 mg; ■, 0.2 mg. The values were based on the protein contents of the cell extract with PqqA, B, D, E, and F (pBCP329). (B) A constant amount of cell extract (0.1 mg of protein) containing PqqC (pBCP390) was combined with various amounts of cell extract containing PqqA, B, D, E, and F (pBCP329): ●, 0.05 mg; ○, 0.1 mg; ■, 0.2 mg. The values are based on the protein contents of the cell extract with PqqC (pBCP390).

Table 4 also shows that extracts lacking PqqC could be complemented with extracts containing PqqC and that extracts lacking PqqB produced PQQ. 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 PQQ reached its maximum within 30 min (Fig. 3B). A plasmid that produced only PqqC, pBCP390, also restored PQQ synthesis. The truncated *pqqD* gene from pBCP390 was not functional, since it could not complement pBCP338 [*pqqABC*(Δ*D*)*EF*] in vivo, the PQQ concentration in the culture supernatant being less than 0.6 nM.

We determined the amount of PQQ 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 PQQ production increased with increasing amounts of PqqC-containing extract when the amounts of PqqA, B, D, E, and F were kept constant. The same amount of PQQ 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 rate of PQQ production increased. The amount of PQQ 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 PQQ biosynthesis which could be converted into PQQ by a PqqC-containing cell extract. This was studied in more detail by measuring the amount of the intermediate and PQQ 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 PQQ.

TABLE 5. PQQ and assayable PQQ biosynthesis intermediate in *E. coli* JA221 and *K. pneumoniae* KA220 cell extracts containing various Pqq proteins[a]

Pqq proteins[b]	Plasmid(s)	Time (min)	Amt produced (pmol/mg of protein)	
			Intermediate	PQQ
E. coli JA221				
—BCDEF	pBCP325	0	<0.4	<0.4
A—CDEF	pBCP324	0	10.2	1.1
		45	<0.4	10.8
A—CDEF	pBCP328	0	6.6	0.6
		45	<0.4	7
AB—DEF	pBCP329	0	16.0	<0.4
AB—DEF + C	pBCP329 + pBCP390	0	9.0	<0.4
		30	<0.4	8.4
ABC—EF	pBCP338	0	<0.4	<0.4
ABCD—F	pBCP330	0	<0.4	<0.4
ABCDE	pBCP186	0	<0.4	0.8
K. pneumoniae KA220				
A—CDEF	pBCP324	0	13.3	0.6
		45	<0.4	13.5

[a] Cell extract (one extract or a combination of extracts) was incubated as described in Materials and Methods. The reaction was stopped by $HClO_4$/KOH treatment at the time indicated (0, 30, or 45 min). After removal of the $KClO_4$ precipitate, the supernatant was assayed for PQQ and assayable PQQ biosynthesis intermediate, as described in Materials and Methods, with apo-GCD. Values are based on the protein contents of the sample, e.g., single extract or the total protein content of the extract lacking PqqC combined with the PqqC-containing extract.

[b] Deletion or inactivation of a particular *pqq* gene is indicated by a dash at the appropriate position.

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 PQQ biosynthesis into the growth medium. The culture supernatant of JA221/pBCP329 [*pqqAB*(*C46*::Tn*5tac1*)*DEF*] incubated with an extract containing only PqqC produced PQQ. The concentration of this intermediate in the supernatant was 2 to 8 nM. Production of the intermediate was also investigated in *K. pneumoniae* KA222, which lacks PqqC, transformed with plasmid pBCP329. The concentration of intermediate in the culture supernatant of *K. pneumoniae* KA222/pBCP329 was 25 to 60 nM. These concentrations of intermediate in the culture supernatant should be compared with the amount of PQQ excreted by *E. coli* JA221/pBCP165 and *K. pneumoniae* NCTC 418/pBCP165, 180 and 540 nM, respectively.

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

amount of PQQ produced was reached after 45 min. A small amount of PQQ was detectable at the start of the experiment (Table 4 and Fig. 3C). Since in pBCP328 the Tn*5tac1* element was inserted in the middle of the *pqqB* gene possibly producing a truncated but still active PqqB protein, pBCP324 [*pqqA*(*B38*::Tn*5tac1*)*CDEF*], in which a Tn*5tac1* 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 PQQ comparable to those in an extract made from cells harboring pBCP328 (Table 4). Addition of an equal amount of a cell extract containing PqqB (pBCP176) to a pBCP328-derived extract resulted in a small stimulation of PQQ production (Table 4) because the Tn*10* insertion in the *pqqC* gene was not completely polar (data not shown). This increase in PQQ production became evident when the amount of PQQ 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 PQQ (9 pmol of PQQ per mg of protein in 30 min).

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

Studies with *E. coli* and *K. pneumoniae* cell extracts lacking PqqB showed that they contained the same intermediate of PQQ biosynthesis as cell extracts from PqqC-lacking cells. In vitro, PQQ production in PqqB-lacking extracts was stopped at different times by the addition of $HClO_4$, and the amount of PQQ and PQQ biosynthesis intermediate in the supernatant (after $KClO_4$ removal) was determined. At the start of the experiment, this supernatant contained very little PQQ (see above), but when PqqC was added, PQQ 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 PQQ (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 PQQ 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 PQQ 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 PQQ 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 PQQ 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 PQQ biosynthesis. Using an in vitro system, we have also detected an intermediate in PQQ biosynthesis, and we have shown that the PqqC protein probably catalyzes the last step in PQQ synthesis.

The role of each of the *K. pneumoniae pqq* genes in PQQ biosynthesis in intact cells and in metabolism via a PQQ-dependent pathway was studied in *E. coli* since *E. coli* can synthesize apo-glucose dehydrogenase, which oxidizes glucose to gluconate, but not its cofactor, PQQ. 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 PQQ 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 PQQ secretion into the medium. It is important to note that the *pqqA* gene complemented in *trans* and was required for PQQ synthesis and excretion. This is in agreement with the hypothesis that the *pqqA* gene encodes the precursor polypeptide for PQQ.

Our data show that almost no PQQ 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 PQQ for the formation of the quinone groups (19, 44). We cannot presently exclude the possibility, however, that one or more enzymes involved in PQQ 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 *pqqA* (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 *pqqA* 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 PQQ biosynthesis, we have developed an in vitro system for PQQ 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 PQQ per mg of protein. Extracts lacking the PqqA, PqqC, PqqD, PqqE, or PqqF protein contained no PQQ or amounts below the detection level (except maybe in the case of PqqF; see below). A certain amount of PQQ was detected in extracts of PqqB-deficient cells, however.

In vitro complementation could be clearly demonstrated in the case of PqqC. PQQ 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 PQQ. These results strongly suggest that an intermediate in PQQ 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 PQQ 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 PQQ-like molecule rather than a polypeptide resembling PqqA. However, it cannot be completely excluded at present that 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 PQQ. At present, we are purifying and characterizing the detected intermediate.

In all other cases, reconstitution of PQQ 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 PQQ 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 PQQ 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 PQQ-dependent glucose dehydrogenase pathway if the *pqqB* gene was lacking. These results point to an essential role for PqqB in PQQ biosynthesis. To our surprise, however, a cell extract, containing all Pqq proteins except PqqB could produce PQQ in vitro in a time-dependent manner. It should be noted, however, that the rate of PQQ 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 PQQ-requiring pathway (15, 17), whereas in the case of *M. extorquens* AM1, it was concluded that the PqqG protein was required for PQQ 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 PQQ, 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 PQQ 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 PQQ across the membrane. However, PqqB could modify an existing transport system so that secretion of PQQ becomes possible. Lack of PqqB could cause accumulation of PQQ 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., PQQ) 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 PQQ, just above the detection limit. These cells also produced some PQQ 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 PQQ 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 PQQ-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 pqqABCDE* 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 PQQ biosynthesis, i.e., produce small amounts of PQQ. 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 PQQ 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 PQQ 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-

coding PqqD was more abundant than the transcripts encoding PqqD and PqqG (the homolog of *K. pneumoniae* PqqB) together (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 *pqqA* 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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