JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 447–455
0021-9193/89/010447-09$02.00/0

Vol. 171, No. 1

# *Acinetobacter calcoaceticus* Genes Involved in Biosynthesis of the Coenzyme Pyrrolo-Quinoline-Quinone: Nucleotide Sequence and Expression in *Escherichia coli* K-12

NORA GOOSEN,* HAROLD P. A. HORSMAN, RENÉ G. M. HUINEN, AND PIETER VAN DE PUTTE
*Department of Molecular Genetics, University of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands*

Received 23 June 1988/Accepted 26 September 1988

**Synthesis of the coenzyme pyrrolo-quinoline-quinone (PQQ) from *Acinetobacter calcoaceticus* requires the products of at least four different genes. In this paper we present the nucleotide sequence of a 5,085-base-pair DNA fragment containing these four genes. Within the DNA fragment three reading frames are present, coding for proteins of $M_r$ 10,800, 29,700, and 43,600 and corresponding to three of the PQQ genes. In the DNA region where the fourth PQQ gene was mapped the largest possible reading frame encodes for a polypeptide of only 24 amino acids. Still, the expression of this region is essential for the biosynthesis of PQQ. A possible role for this DNA region is discussed. Sandwiched between two PQQ genes an additional reading frame is present, coding for a protein of $M_r$ 33,600. This gene, which is probably transcribed in the same operon as three of the PQQ genes, seems not required for PQQ synthesis. Expression of the PQQ genes in *Acinetobacter lwoffi* and *Escherichia coli* K-12 led to the synthesis of the coenzyme in these organisms.**

Pyrrolo-quinoline-quinone (PQQ) is used by a variety of organisms as cofactor in dehydrogenase reactions. In *Pseudomonas* species and *Acinetobacter calcoaceticus* PQQ is associated with glucose dehydrogenase (GDH) (1, 5, 22). In *Pseudomonas testosteroni* (7) and *Pseudomonas stutzeri* (M. van Kleef, personal communication) and PQQ-dependent alcohol dehydrogenase is present. *Thiobacillus versutus* (19) and *Paracoccus denitrificans* (2) synthesize methylamine dehydrogenase, to which PQQ is covalently linked.

Also in mammals the presence of PQQ as cofactor has been demonstrated. In bovine plasma (10, 15) and porcine kidney (20) PQQ-dependent amine oxidases have been identified, and it has been suggested (4, 15) that amine oxidases from other mammals, *Saccharomyces cerevisiae*, *Aspergillus*, and plants also use PQQ as a coenzyme.

In some bacterial species, viz., *A. calcoaceticus*, PQQ is synthesized constitutively in the absence of the enzyme's substrate (i.e., glucose), whereas in *P. stutzeri* PQQ can be detected only when ethanol is present in the culture medium as an inducing agent (M. van Kleef, personal communication). *Escherichia coli* K-12 and *Acinetobacter lwoffi* do not produce PQQ. These organisms synthesize the apo-GDH enzyme and are dependent on uptake of PQQ from the culture medium for the constitution of the holo-GDH (8, 21).

The biosynthetic pathway of PQQ has not yet been elucidated. Our approach to this problem was to clone the genes and to study the encoded gene products that are involved in PQQ synthesis. Recently we reported the isolation of a plasmid containing four cloned PQQ genes from *A. calcoaceticus* on a 5,000-base-pair (bp) fragment (6). In this paper we report the complete nucleotide sequence of this fragment and show that the genes from *A. calcoaceticus* can direct the synthesis of the cofactor in other microorganisms.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The following bacterial strains have been previously described: *E. coli* JM101 (Δ*lac-pro thi* F'*traD36 proAB lacI*$^q$ZΔM15) (13), *E. coli* PPA41 (*thi ptsI*) (8), and *A. lwoffi* (21). These strains were cultured in L broth, on L plates, or in defined minimal medium (14) as indicated. Bacterial matings were performed as described previously (6). Acid production by *A. lwoffi* was tested on L plates containing 0.4% glucose and a few drops of phenol red. Acid production resulted in yellow plates, whereas the plates of non-acid-producing strains remained red.

**Chemicals and reagents.** Restriction endonucleases were from Bethesda Research Laboratories, Inc. T4 DNA ligase and DNA polymerase (Klenow enzyme) were from Pharmacia Fine Chemicals. All recombinant DNA techniques were essentially as described previously (12). Purified PQQ was a gift from M. van Kleef. The universal primer for DNA sequencing (5'-GTAAAACGACGGCCAGT-3') was purchased from Pharmacia. The oligonucleotide primer corresponding to the Tn*5* end sequences (5'-GGTTCCGTTCAG GACGCTAC-3') was kindly synthesized by J. H. van Boom. Radiochemicals and in vitro coupled transcription-translation kits were from Amersham Corp.

**DNA sequencing.** Transformation of strain JM101 was performed as described previously (13). M13 phage particles were purified, and the single-stranded DNA was extracted by the method of Sanger et al. (16). For the sequencing reaction the primer extension method (17) was used. Sequences were compiled and analyzed by using the sequence analysis software package of the University of Wisconsin Genetics Computer Group (3).

**Sequence strategy.** A schematic representation of the sequence strategy is indicated in Fig. 1. The *Eco*RI-*Bam*HI fragment of pSS2, containing the four PQQ genes, was subcloned in M13mp19, resulting in pSS144. Deletion derivatives for DNA sequencing were isolated by an adaptation of the method of Hong (9). pSS114 was partially digested with *Sau*3A, *Rsa*I, or *Dra*I. Linear fragments were isolated, digested with *Bam*HI (for *Sau*3A) or *Hin*cII (for *Rsa*I and *Dra*I), recircularized, and transformed to JM101. Thus the different *Sau*3A, *Rsa*I, and *Dra*I sites were brought close to the primer for sequencing.

For sequencing of the opposing strand, different subclones

* Corresponding author.

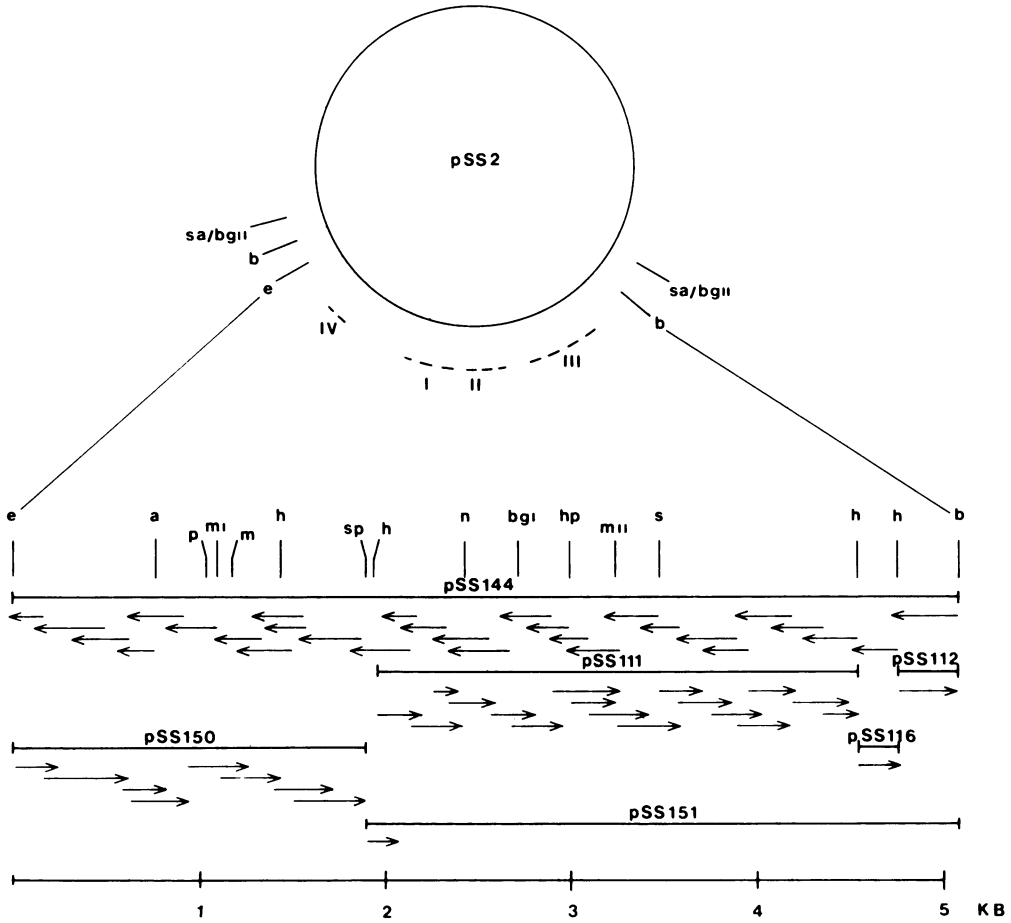


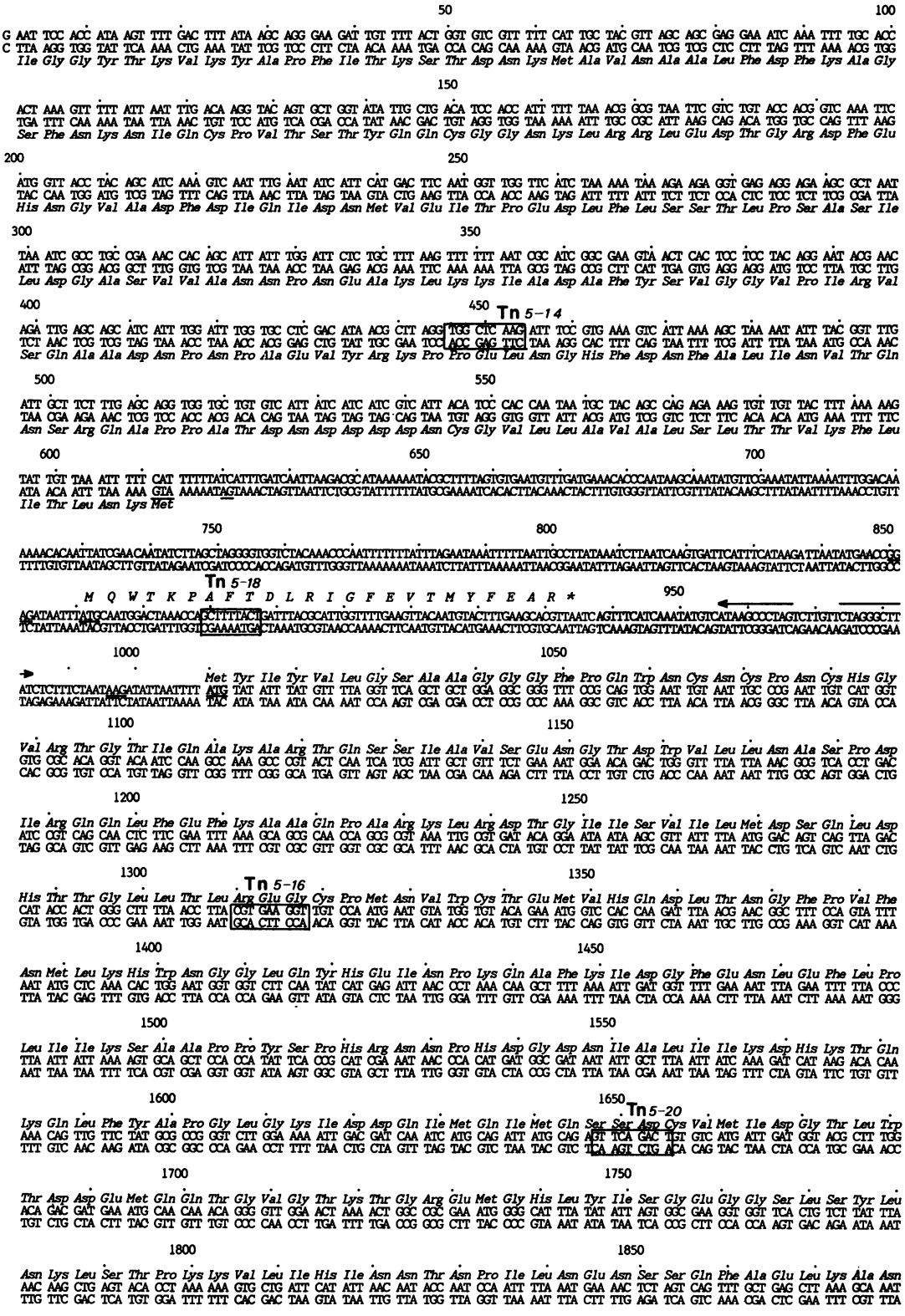

FIG. 1. Schematic representation of the sequencing strategy. Plasmid pSS2, containing a 5.5-kb *Sau*3A insert in the *Bgl*II site of vector pRK290, has been described previously (6). Only restriction sites that occur with low frequency are indicated: a, *Acc*I; b, *Bam*HI; bgi, *Bgl*I; bgii, *Bgl*II; e, *Eco*RI; h, *Hin*dIII; hp, *Hpa*I; m, *Mlu*I; mi, *Mst*I; mii, *Mst*II; n, *Nco*I; p, *Pvu*II; s, *Sac*I; sa, *Sau*3A; sp, *Sph*I. pSS144 contains the *Eco*RI-*Bam*HI fragment in M13mp19, pSS111 contains the *Hin*dIII fragment in M13mp19, pSS112 has the *Hin*dIII-*Bam*HI fragment inserted in M13mp18, pSS150 has the *Eco*RI(*Sal*I)-*Sph*I fragment in M13mp19, pSS116 contains the *Hin*dIII fragment in M13mp18, and pSS151 carries the *Sph*I-*Bam*HI fragment in M13mp18. The arrows show the location, direction, and length of nucleotide sequence obtained from each template.

were constructed. First the internal 2,600-bp *Hin*dIII fragment of pSS2 was cloned into M13mp19, the correct orientation was selected (pSS111), and deletion derivatives were isolated as described above. A *Sal*I linker fragment was inserted into the *Eco*RI site of pSS2, and then the 1,900-bp *Sal*I-*Sph*I fragment was subcloned in M13mp19 (pSS150). Also from pSS150 deletions were constructed by the method described above. The remaining right part of the *Eco*RI-*Bam*HI fragment was sequenced by subcloning the 210-bp *Hin*dIII fragment and the 320-bp *Hin*dIII-*Bam*HI fragment in M13mp18 (pSS116 and pSS112, respectively). Finally, the gap between *Sph*I and *Hin*dIII was sequenced by insertion of the *Sph*I-*Bam*HI fragment in M13mp18 (pSS151).

## RESULTS

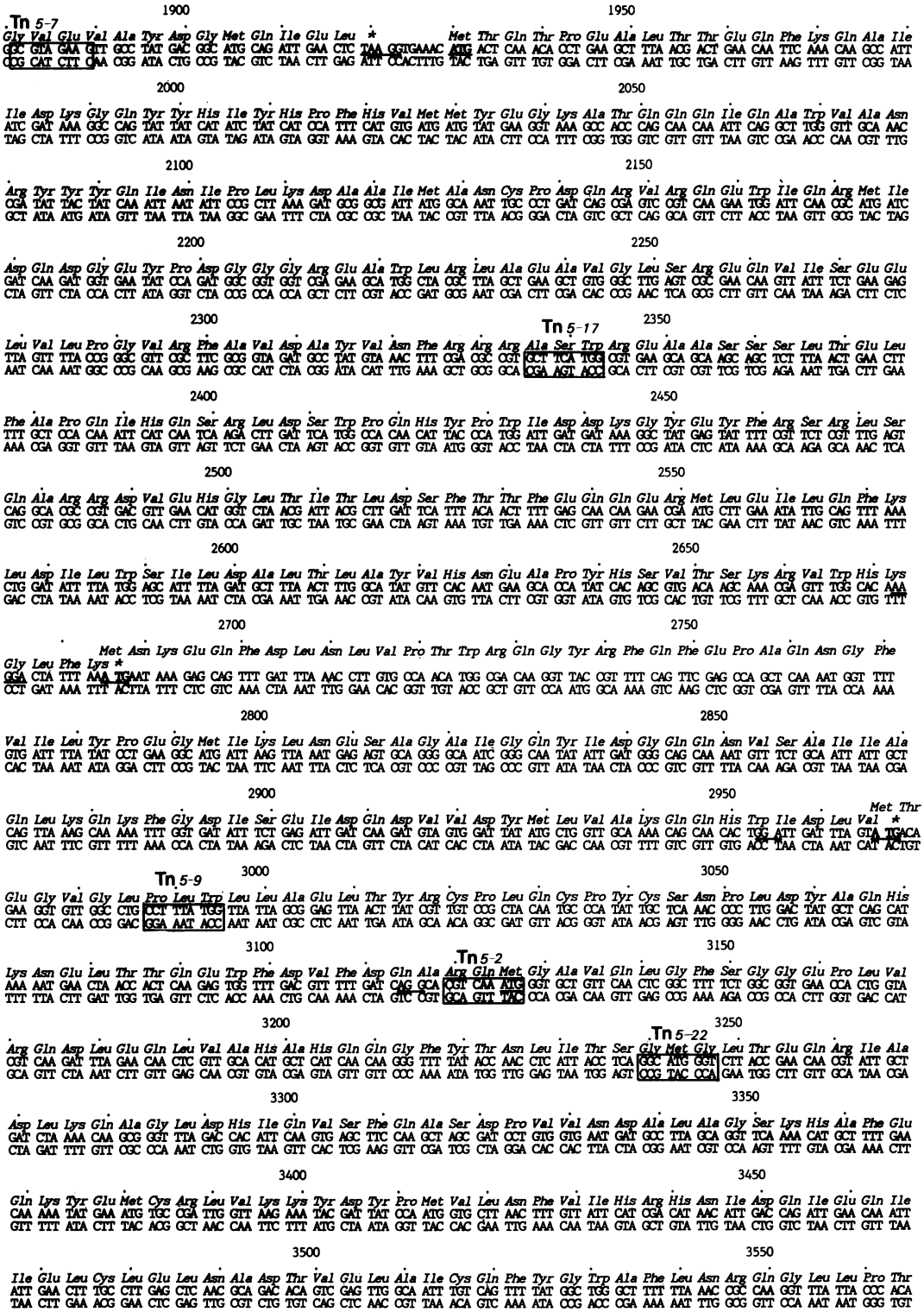
**Nucleotide sequence of the PQQ genes.** Plasmid pSS2 contains a 5.5-kilobase insert from the *A. calcoaceticus* chromosome which complements four different classes of chromosomal PQQ$^-$ mutants and which is therefore expected to carry four different genes involved in PQQ synthesis (6). With Tn*5* insertions these cloned genes (I, II, III, and IV) were approximately mapped within a 5-kilobase *Eco*RI-*Bam*HI fragment (6). We determined the complete nucleotide sequence of this fragment as described in Materials and Methods. It was comprised of 5,087 bp and showed a rather high A+T content (61%). Within the sequenced fragment different open reading frames (ORFs) were indicated. The complete nucleotide sequence and the deduced amino acid sequence of the ORFs are reported in Fig. 2, and a schematic representation of the ORFs is given in Fig. 3.

To determine whether these ORFs correspond to the different PQQ genes, we also determined the precise insertion points of the Tn*5* insertions. Therefore the pSS2-Tn*5* plasmids were restricted with *Eco*RI and *Pst*I. Since *Eco*RI cuts at the left border of the 5-kilobase fragment (Fig. 1) and *Pst*I cuts within Tn*5*, a fragment was generated containing one end of Tn*5* and the PQQ sequences located to the left of the insertion point. This fragment was inserted into M13mp18, and the fusion point was sequenced by using a synthetic oligonucleotide (5′-GGTTCCGTTCAGGACGC TAC-3′) that was complementary to the end of Tn*5* as a primer. As a control also the *Bam*HI-*Pst*I fragment containing the other end of Tn*5* fused to PQQ sequences located to the right of the insertion point was cloned and sequenced. In




this way the insertion points of nine different Tn*5* insertions were determined (Fig. 2). The phenotypes of the pSS2 plasmids containing these Tn*5* insertions (13) are listed in Table 1.

Transposon insertions Tn*5*-16, Tn*5*-20, and Tn*5*-7 were located within an ORF (indicated as gene V) starting with ATG (position 1010) and ending with TAA (position 1921), which could code for a protein of $M_r$ 33,600. It has been shown that chromosomal deletion mutants that lack the complete PQQ region (including gene V) can still be complemented by these Tn*5* insertions (13). Apparently gene V is not involved in PQQ biosynthesis.




Transposon insertion Tn*5*-17 inhibited the expression of both gene I and gene II (Table 1). We have suggested (6) that these two genes are located in the same operon and that, due to a polar effect on transcription, Tn*5* affects expression of both genes. Indeed in the DNA sequence two ORFs were indicated that were closely linked. The first ORF (gene I) started at ATG (position 1930), ended at TGA (position 2688), and coded for a protein of $M_r$ 29,700. Insertion Tn*5*-17 was located within this ORF. The stop codon (TGA) of gene I overlapped with the start ATG (position 2685) of a second ORF (gene II) that stopped at TGA (position 2969), coding for a protein of $M_r$ 10,800. This overlap of start and stop

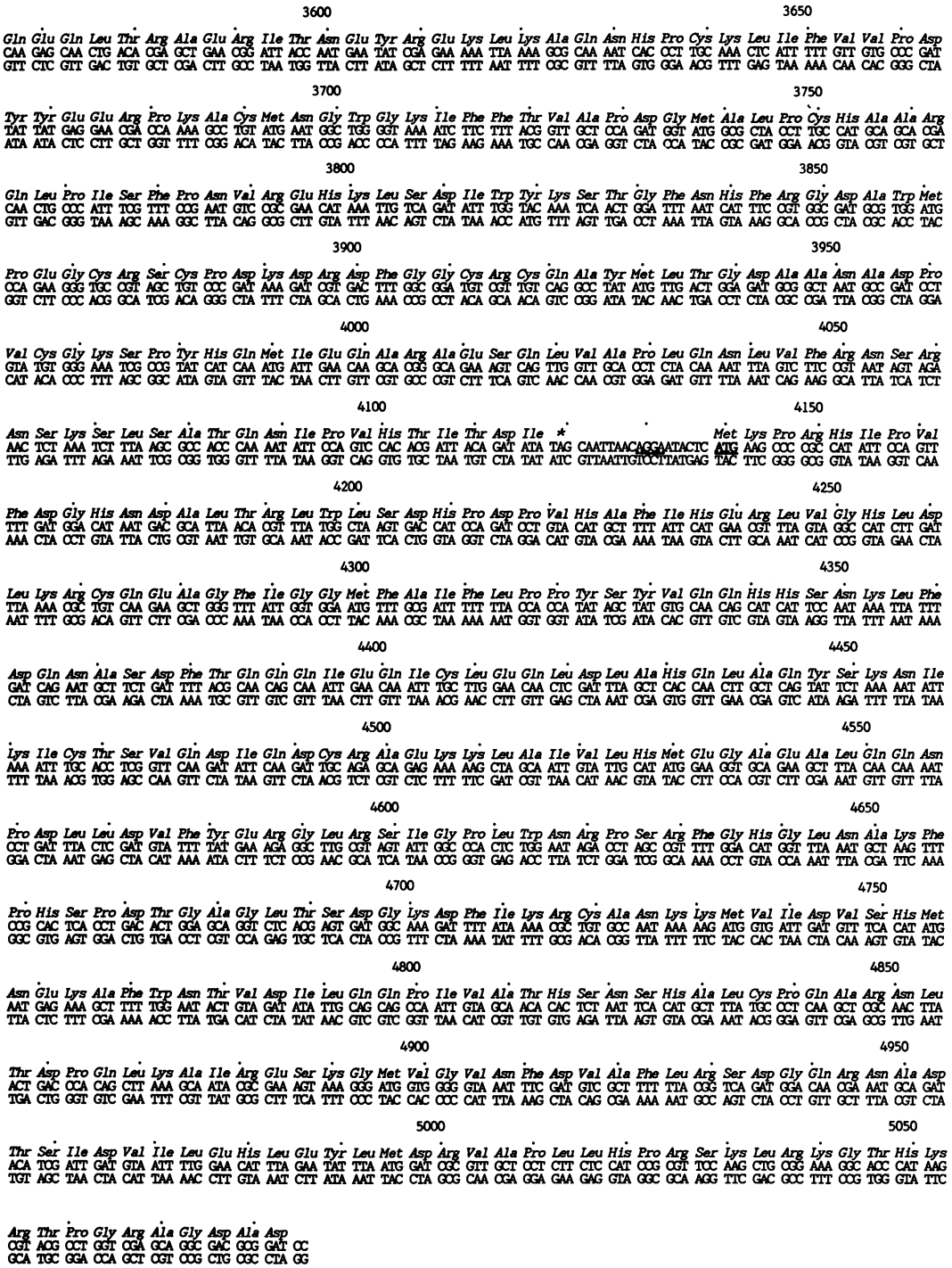


FIG. 2. Sequence of the 5,085-nucleotide *Eco*RI-*Bam*HI insert of pSS2. The predicted amino acid sequence of the different reading frames is indicated as follows (positions): gene L (613 through 1), gene V (1010 through 1921), gene I (1930 through 2688), gene II (2685 through 2969), gene III (2966 through 4118 or 3125 through 4118), and gene R (4139 through 5085). The putative 24-amino-acid polypeptide coded for by gene IV is indicated by one-letter symbols (positions 861 through 935). The ATG translation start sites and the putative ribosome-binding sites are underlined. The insertion sites of the different Tn*5* insertions are indicated by a box, representing the 9 bp that are duplicated upon Tn*5* insertion.

codons suggests that the polar effect that Tn*5*-17 exerted on the expression of gene II might also be due to a coupled translation of both genes.

The stop codon of gene II again overlapped with a putative start ATG (position 2966), followed by an ORF ending with TAG (position 4118) which could code for a protein of $M_r$ 43,600. This ORF might correspond to gene III, since Tn*5*-2 and Tn*5*-22, which abolished gene III expression, were located within this reading frame. However Tn*5*-9, which was mapped 20 bp downstream of this putative start of gene

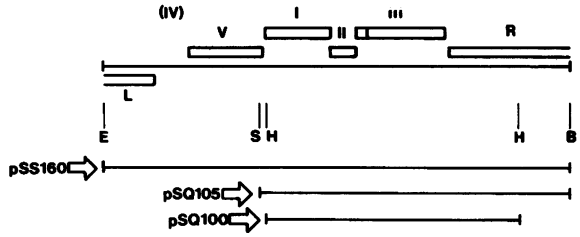



FIG. 3. Schematic representation of the different ORFs (indicated by bars) present in the 5,085-bp *Eco*RI-*Bam*HI fragment. The gene IV region is indicated with brackets. Relevant restriction sites: E, *Eco*RI; S, *Sph*I; H, *Hin*dIII; B, *Bam*HI. The inserts of the plasmids used for analysis of the proteins encoded by the PQQ genes are shown. pSS160 contains the *Eco*RI-*Bam*HI fragment inserted in pUC12, pSQ100 contains the *Hin*dIII fragment inserted in pUC12, and pSQ105 contains the *Sph*I-*Bam*HI fragment inserted in pUC19. The arrows represent the P *lac* promoter from which the PQQ genes are expressed.

TABLE 1. Complementation of different PQQ$^-$ mutants by Tn*5* insertion plasmids (6)

| Tn*5* insertion | Complementation of PQQ$^-$ mutant | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Tn*5*-2 | + | + | − | + |
| Tn*5*-7 | + | + | + | + |
| Tn*5*-9 | + | + | + | + |
| Tn*5*-14 | + | + | + | + |
| Tn*5*-16 | + | + | + | + |
| Tn*5*-17 | − | − | + | + |
| Tn*5*-18 | + | + | + | − |
| Tn*5*-20 | + | + | + | + |
| Tn*5*-22 | + | + | − | + |

III (Fig. 2), did not affect PQQ expression. An alternative ATG start could be indicated at position 3125 from which a protein of $M_r$ 37,400 could be synthesized. If this second ATG were used for gene III translation, there would be an intracistronic region of 150 bp between genes II and III. To test whether a promoter for gene III expression was located within this region we isolated two deletion derivatives of pSS2 containing Tn*5*-9. First, all the sequences to the left of Tn*5*-9 were deleted (Fig. 4a). The resulting plasmid (Tn*5*-9/*Bgl*II) still complemented gene III mutants, indicating that no sequences to the left of Tn*5*-9 are essential for gene III expression. Then 14 bp to the right of Tn*5*-9 were deleted (Fig. 4a). Complementation studies of the resulting plasmid (Tn*5*-9/*Hpa*I) showed that as a result of this deletion gene III was no longer expressed. A possible explanation is that the promoter (or part of the promoter) for gene III was located within these 14 bp of the PQQ sequence. However, since in Tn*5*-9/*Hpa*I also most of the Tn*5* insertion had been deleted (Fig. 4a), it is more likely that a promoter was present in the inverted repeat of Tn*5*-9 which directed expression of gene III and which was deleted in Tn*5*-9/*Hpa*I. Evidence for a promoter in the inverted repeats of Tn*5* from which transcription can proceed into sequences adjacent to the Tn*5* insertion has already been obtained in *E. coli* (11). This promoter was mapped between the *Bal*I and *Hpa*I sites of the inverted repeat (Fig. 3). Recently, by subcloning the *gdh* gene of *A. calcoaceticus* we obtained evidence that this gene can be expressed in this organism from a promoter located within Tn*5* (A. M. Cleton-Jansen et al., manuscript in preparation).

If indeed the Tn*5* promoter is also active in *A. calcoaceticus*, the presence of a Tn*5* insertion could be nonpolar for transcription, since all genes downstream from the Tn*5* insertion could be expressed under the control of this promoter. In this case it is possible that genes I, II, and III are located in one operon. The presence of Tn*5*-17 in gene I might block expression of gene II by a polar effect on translation, whereas gene III could still be expressed under the control of the Tn*5* promoter.

If the second ATG at position 3125 were the correct start site for gene III translation, Tn*5*-2 would be located between the ribosomal binding site and the translation start site (Fig. 2). The deficiency in gene III expression in this case would then probably be due to an inhibition of translation initiation.

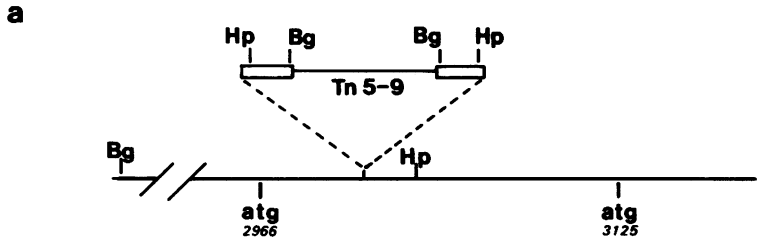




FIG. 4. (a) Schematic representation of the translation start region of gene III. The possible translation start sites for gene III are ATG (position 2966) and ATG (position 3125). The position of Tn*5*-9 is shown. Tn*5*-9/*Bgl*II was constructed by deletion of the *Bgl*II fragment (*Bgl*II cuts to the left of the 5,085-bp insert and near the inside ends of the inverted repeats of Tn*5*). Tn*5*-9/*Hpa*I was constructed by deletion of the *Hpa*I fragment (*Hpa*I cuts 14 bp to the right of Tn*5*-9 and 190 bp from the outside ends of the inverted repeats of Tn*5*). Relevant restriction sites: Bg, *Bgl*II; Hp, *Hpa*I. (b) Nucleotide sequence of the junction between Tn*5*-9 and the gene III region of pSS2. Base 2987 is the first nucleotide of the PQQ sequence. Putative ribosomal binding sites and the translation start site within the Tn*5* sequence are underlined. The amino acids of the proposed fusion protein are shown.

However, arguments could be given for the first ATG at position 2966 being the correct start codon. Examination of the nucleotide sequence of the Tn*5* region that is transcribed by the putative Tn*5* promoter (Fig. 4b) revealed the presence of an ATG codon that was preceded by a good Shine and Dalgarno sequence (5'-AGGAGGT-3') and followed by an ORF that continued until the end of the transposon. As far as we know, no evidence in the literature has been presented that this sequence can serve as a translation start site, but if it does so in *A. calcoaceticus* the presence of Tn*5*-9 might generate a fusion protein (Fig. 4b). This fusion protein should contain 22 amino acids from Tn*5* and the amino acids of the gene III product starting with Pro (position 2987). If the ATG at position 2966 were the correct start site for gene III translation, the fusion protein would lack only the seven N-terminal amino acids of the gene III product and would therefore be very likely to retain activity. In conclusion, our results still leave two possible translation start sites for gene III, one generating a protein of $M_r$ 37,400 and one generating a protein of $M_r$ 43,600.

For the mapping of the gene corresponding to the fourth complementation group in PQQ synthesis (gene IV), the position of Tn*5*-18, which blocks the expression of this gene (Table 1), was determined. Surprisingly, only two very small ORFs were indicated in this region. One ORF started with ATG (position 861) and ended with TAA (position 933), which would code for a protein of 24 amino acids (Fig. 2). The second ORF was located in the opposing strand starting with ATG (position 898) ending with TGA (position 832) and coded for a protein of 22 amino acids. Even if we consider the unlikely possibility that start sites other than ATG, GTG, or TTG are used in *A. calcoaceticus*, the largest possible ORF is from ACC (position 846) to TAG (position 965), which would code for a protein of 39 amino acids. Tn*5*-18 could also block expression of gene IV if it is not located within the ORF but in the leader of the transcript of gene IV. If we consider the ORFs of more considerable length near Tn*5*-18, however, this possibility also seems very unlikely. The first ORF to the right of Tn*5*-18 was the ORF of gene V, which was not involved in PQQ expression (see above). The only considerable ORF located to the left of Tn*5*-18 started with ATG (position 613) and proceeded until the *Eco*RI site. Within this reading frame (indicated as gene L) Tn*5*-14 was mapped (Fig. 2). Since Tn*5*-14 did not affect the complementation of PQQ$^-$ mutants (Table 1), gene L also is not involved in PQQ synthesis. Thus, the effect of Tn*5*-18 on PQQ synthesis cannot be due to an influence on expression of these larger ORFs. Since Tn*5*-18 did not affect the expression of genes I, II, and III (Table 1), the conclusion seems justified that the DNA region in which Tn*5*-18 is located has to code for a product that plays a role in PQQ synthesis. A closer examination of the gene IV DNA region revealed the presence of a G+C-rich hairpin structure (positions 956 through 984) followed by an A+T-rich stretch of DNA. This structure is very likely to be a terminator for transcription initiated within or to the left of the gene IV region. Two possibilities for the gene IV product can now be considered. First the RNA that terminates at the hairpin structure might code for the 24-amino-acid polypeptide starting at ATG (position 861). Second, the RNA itself might play a role in PQQ synthesis. In both cases an unusual pathway for PQQ biosynthesis can be expected.

The last ORF present in the sequence (indicated as gene R) started at position 4139 (ATG) and proceeded beyond the *Bam*HI site. Since no other classes of PQQ mutants like the four classes described above have been found (6), this reading frame is not likely to play a role in PQQ synthesis.

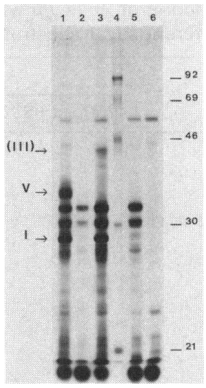



FIG. 5. Autoradiogram of [$^{35}$S]methionine-labeled proteins produced by in vitro transcription-translation of different DNA templates. Lanes: 1, pSS160; 2, pSQ100; 3, pSQ105; 4, markers; 5, pUC18; 6, no DNA. The positions of the marker bands are indicated. The arrows represent the protein bands corresponding to translation products of genes I, III, and V.

**Analysis of the proteins encoded by the PQQ genes.** To test whether proteins corresponding to the different ORFs could be detected, we used an in vitro coupled transcription-translation system derived from *E. coli*. When pSS2 was used as template in this system, no specific proteins could be detected. This could be due to an improper recognition of *A. calcoaceticus* promoters by *E. coli* RNA polymerase. Therefore we placed the PQQ genes under control of the *E. coli lac* promoter by cloning the 5,085-bp *Eco*RI-*Bam*HI fragment of pSS2 in pUC12, resulting in pSS160. The autoradiogram of the labeled proteins produced by this plasmid and run on a 12% polyacrylamide gel is shown in Fig. 5. Two specific protein bands that were not produced when the vector pUC12 was used as template could be detected. The first had a molecular weight of about 34,000, which might very well correspond to the product of gene V ($M_r$ 33,600). The second protein band migrated at the position of approximately $M_r$ 29,000 and is therefore likely to be the product of gene I ($M_r$ 29,700). To test whether these protein bands indeed correspond to the translation products of genes I and V, we constructed two other plasmids. pSQ105 contained the 3,175-bp *Sph*I-*Bam*HI fragment in pUC19 (Fig. 4) and was expected to express genes I, II, and III under control of the *lac* promoter. pSQ100 contained the 2,600-bp *Hin*dIII fragment in pUC12 (Fig. 4). Since *Hin*dIII cuts 15 bp downstream the postulated translation start of gene I, this plasmid was expected to express only genes II and III. Indeed, the $M_r$ 34,000 protein band (gene V) was missing when both plasmids were translated (Fig. 5). The $M_r$ 29,000 band was still present in pSQ105 but absent in pSQ100. So the $M_r$ 29,000 protein was very likely to be the translation product of gene I, and the postulated translation start of this gene indeed was located between the *Sph*I and *Hin*dIII sites.

Because the translation product of gene II was very small ($M_r$ 10,800), no band corresponding to this protein is visible in Fig. 5. The protein of gene III, however, should be large enough to be detectable. We have shown above that two possible starts for gene III translation can be considered, one resulting in a protein of $M_r$ 37,400 and the other resulting in a protein of $M_r$ 43,600. With pSQ105 a weak protein band of about $M_r$ 44,000 was visible. If this band corresponded to the gene III product, this would mean that translation of gene III

TABLE 2. Acid production of *A. lwoffi* with or without pSS2 on different sugar substrates

| Substrate | Acid production | |
|---|---|---|
| | Without pSS2 | With pSS2 |
| Glucose | − | + |
| Glucose + PQQ | + | + |
| Galactose | − | + |
| Galactose + PQQ | + | + |
| Arabinose | − | + |
| Arabinose + PQQ | + | + |
| Xylose | − | + |
| Xylose + PQQ | + | + |
| Lactose | − | − |
| Lactose + PQQ | − | − |

starts with the first ATG (position 2966). However, the gene III translation product was not observed when pSS160 or pSQ100 were used as a template. Possibly the expression of gene III, which is already very low in pSQ105, is for an unknown reason even more reduced in the other two plasmids.

**Expression of the PQQ genes in *A. lwoffi* and *E. coli* K-12.** *A. lwoffi* and *E. coli* K-12 produce a PQQ-dependent GDH (8, 21). However, these bacterial species do not synthesize PQQ. Therefore the holo-GDH can be formed only when PQQ is supplied from the culture medium. To test whether our four cloned PQQ genes contain sufficient information for the biosynthesis of PQQ, we introduced these genes into *A. lwoffi* and *E. coli* and tested whether these bacteria produced an active GDH enzyme. First pSS2 was introduced in *A. lwoffi* by conjugation, and then the transconjugants were tested for acid production on different sugar substrates as described in Materials and Methods. The introduction of pSS2 in *A. lwoffi* resulted in acid production when GDH-specific sugars were used as substrates (Table 2). This indicates that in the presence of pSS2 a sufficient amount of PQQ is synthesized for the reconstitution of the holo-GDH enzyme. To test whether *A. lwoffi* is normally lacking all the PQQ enzymes and not just carrying a mutation in one of the PQQ genes, we also investigated whether the pSS2 plasmids with the different Tn*5* insertions produced PQQ. *A. lwoffi* with pSS2 carrying the insertion Tn*5*-2, Tn*5*-17, or Tn*5*-18 did not produce acid on GDH-specific sugars, whereas upon introduction of pSS2 with Tn*5*-7, Tn*5*-9, Tn*5*-16, or Tn*5*-20 acid production due to GDH activity could be shown. Apparently all four genes are required for PQQ synthesis in *A. lwoffi*.

Next we tested whether also *E. coli* K-12 could synthesize PQQ upon introduction of the four genes. For this experiment an *E. coli* K-12 strain carrying a *ptsI* mutation (PPA41) was used. Due to the *ptsI* mutation, this strain no longer grows on glucose as the sole carbon source. In the presence of PQQ, however, the GDH enzyme can be used, and growth on glucose is restored (8). Previously we have shown (6) that PPA41 carrying pSS2 still does not grow on minimal medium plates with glucose. Since this might be due to an improper expression of the PQQ genes by the *E. coli* RNA polymerase, we also transformed pSS160 (carrying the PQQ genes under control of the *lac* promoter) to PPA41. The resulting transformants did form colonies on minimal medium plates with glucose after incubation at 37°C for 2 days. No growth was observed on control plates containing mannitol as the carbon source, indicating that the transformants were still mutated in *ptsI* and that growth on glucose had to be the consequence of PQQ synthesis. The PQQ production, however, was apparently low, since in the presence of additional PQQ in the culture medium colony formation on glucose plates was already observed after incubation for 1 day. We showed above that at least the in vitro synthesis of the gene III protein with *E. coli* extracts was very low, which could be the reason for the low level of PQQ production in vivo. To test whether in *E. coli* the expression of genes I, II, and III is sufficient for PQQ production, we also introduced pSQ105 (containing these three genes under control of the *lac* promoter) into PPA41. The resulting transformants did not form colonies on minimal medium plates with glucose even after prolonged incubation, meaning that also in *E. coli* the product of gene IV (which might be an RNA or a small polypeptide) is essential in the biosynthetic pathway of PQQ.

## DISCUSSION

Biosynthesis of the coenzyme PQQ is thought to be a multistep process involving at least five or six different enzymes. In this paper we have shown that expression of four different PQQ genes from *A. calcoaceticus* in *E. coli* or *A. lwoffi* is sufficient for the synthesis of the coenzyme in these organisms. Sequence analysis of the PQQ genes showed that only three of them (genes I, II, and III) code for proteins of a size ($M_r$ 29,700, 10,800, and 43,600, respectively) that can be expected for proteins with an enzymatic function. The fourth gene (gene IV), however, does not seem to code for an enzyme, since the most probable reading frame comprises only 24 amino acids. An explanation for the presence of so few PQQ-specific enzymes might be that a precursor resembling the mature coenzyme structure is already present, not only in *A. calcoaceticus* but also in *A. lwoffi* and *E. coli*.

The intriguing question is what the role of gene IV in PQQ synthesis might be. Complementation studies have shown that the gene IV region is coding for a *trans*-acting product. Since among 40 independently isolated PQQ$^-$ mutants of *A. calcoaceticus* no gene IV point mutations were found (6; unpublished data), this product was already predicted to be relatively small. The 3′ end of the gene IV region contains a G+C-rich hairpin followed by an A+T-rich stretch of DNA, which is characteristic of a transcription terminator. This suggests that a small transcript complementary to only gene IV DNA is formed. Two possible functions for this gene IV RNA can be considered.

First, the gene IV RNA itself might in some way play a role in PQQ synthesis. We consider it unlikely that this RNA would play a direct structural role, but a function as a positive regulator cannot be excluded. RNA has been shown to act in *trans* as a negative regulator in the replication of plasmid ColE1 (18). However until now it has not been reported that RNA can also act as a positive regulator.

As a second possibility the gene IV RNA might be translated in a 24-amino-acid polypeptide, which in turn might have a function in PQQ synthesis. Recently it has been shown that the amino acids tyrosine and glutamate are used as precursors for PQQ biosynthesis in *Hyphomicrobium* sp. strain X (20a) and *Methylobacterium* sp. strain AM1 (C. J. Unkefer, personal communication). Since both amino acids are present in the 24-amino-acid polypeptide, it is possible that this polypeptide is used as a precursor substrate. Through the tertiary structure of the polypeptide both amino acids could be brought together, the enzymes could make the bonds, and finally the complete (or almost complete)

PQQ molecule could be cut out of the polypeptide. Still, when such a polypeptide is used as a precursor it remains unlikely that only three enzymes are sufficient for the complete synthesis of PQQ, since the chemical bonds that have to be made will require multiple enzymatic steps. However, enzymes could be used that also play a role in other biochemical processes.

Finally, the 24-amino-acid polypeptide might also play a role in the transport of PQQ across the cytoplasmic membrane. The active site of the PQQ-dependent GDH enzyme is located at the periplasmic side of the inner membrane (4). Therefore eventually also the PQQ has to be present in the periplasmic space. This could be achieved in two ways. First, the PQQ enzymes could be transported through the inner membrane, and the PQQ could be synthesized in the periplasm. The predicted amino acid sequences of the different PQQ enzymes, however, do not reveal the presence of N-terminal signal peptides (a stretch of hydrophobic amino acids flanked by hydrophilic residues). Alternatively, the PQQ could be synthesized in the cytoplasm and subsequently translocated to the periplasm. The 24-amino-acid polypeptide might be involved in this process, although it also is not highly hydrophobic.

Besides the putative transcription terminator of gene IV, no transcription terminator signals seem to be present at the end of the other PQQ genes. Moreover, the sequence data have shown that the translation start of gene II overlaps with the end of gene I, the start of gene III might overlap with the end of gene II, and the ORFs of genes V and I are separated by only 8 bp. Taken together, these results indicate that it is very likely that genes V, I, II, and III are transcribed from one promoter (although it is still possible that gene III has its own promoter). Gene V, however, which would be the first gene in this operon, is not involved in the synthesis of PQQ. Gene V also does not play a role in the transport of electrons from PQQ to the electron transport chain, since deletion mutants lacking the complete PQQ region (including gene V) show normal GDH activity upon addition of PQQ (13). A reason for the concerted expression of gene V and the PQQ genes might be that gene V codes for another PQQ-dependent enzyme.

## ACKNOWLEDGMENTS

We thank Mario van Kleef for fruitful discussions.

Part of this work was supported by the Netherlands Technology Foundation (S.T.W.).

## LITERATURE CITED

1. **Ameyama, M., K. Matsushita, Y. Ohno, E. Shinegawa, and E. Adachi.** 1981. Existence of a novel prosthetic group, PQQ, in membrane-bound electron transport chain-linked, primary dehydrogenases of oxidative bacteria. FEBS Lett. **130**:179–183.
2. **Davidson, V. L., and J. W. Neher.** 1987. Evidence for two subclasses of methylamine dehydrogenases with distinct large subunits and conserved PQQ-bearing small subunits. FEMS Microbiol. Lett. **44**:121–124.
3. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
4. **Duine, J. A., J. Frank, and J. A. Jongejan.** 1986. PQQ and quinoprotein enzymes in microbial oxidations. FEMS Microbiol. Rev. **32**:165–178.
5. **Duine, J. A., J. Frank, and J. K. van Zeeland.** 1979. Glucose dehydrogenase from *Acinetobacter calcoaceticus*: a quinoprotein. FEBS Lett. **108**:443–446.
6. **Goosen, N., D. A. M. Vermaas, and P. van de Putte.** 1987. Cloning of the genes involved in synthesis of coenzyme pyrroloquinoline-quinone from *Acinetobacter calcoaceticus*. J. Bacteriol. **169**:303–307.
7. **Groen, B. W., M. A. G. van Kleef, and J. A. Duine.** 1986. Quinohaemoprotein alcohol dehydrogenase apoenzyme from *Pseudomonas testosteroni*. Biochem. J. **234**:611–615.
8. **Hommes, R. W. J., P. W. Postma, O. M. Neyssel, D. W. Tempest, P. Dokter, and J. A. Duine.** 1984. Evidence of a quinoprotein glucose dehydrogenase apoenzyme in several strains of *Escherichia coli*. FEMS Microbiol. Lett. **24**:329–333.
9. **Hong, G. F.** 1982. A systematic DNA sequencing strategy. J. Mol. Biol. **158**:539–549.
10. **Lobenstein-Verbeek, C. L., J. A. Jongejan, J. Frank and J. A. Duine.** 1984. Bovine serum amine oxidase: a mammalian enzyme having covalently-bound PQQ as prosthetic group. FEBS Lett. **170**:305–309.
11. **Lupski, J. R., S. J. Projan, L. S. Ozaki, and G. N. Godson.** 1986. A temperature-dependent pBR322 copy number mutant resulting from a Tn5 position effect. Proc. Natl. Acad. Sci. USA **83**: 7381–7385.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Moog, R. S., M. A. McGuirl, C. E. Cote, and D. M. Dooley.** 1986. Evidence for methoxatin (pyrroloquinolinequinone) as the cofactor in bovine plasma amine oxidase from resonance Raman spectroscopy. Proc. Natl. Acad. Sci. USA **83**:8435–8439.
16. **Sanger, F., A. R. Coulson, B. G. Barrell, and A. J. H. Smith.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143**:161–178.
17. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
18. **Tomizawa, J., and T. Itoh.** 1981. Plasmid ColE1 incompatability determined by interaction of RNAI with primer transcript. Proc. Natl. Acad. Sci. USA **78**:6096–6100.
19. **Van der Meer, R. A., J. A. Jongejan, and J. A. Duine.** 1987. Phenylhydrazine as probe for cofactor identification in amine oxidoreductases. FEBS Lett. **221**:299–304.
20. **Van der Meer, R. A., J. A. Jongejan, J. Frank, and J. A. Duine.** 1986. Hydrazone formation of 2,4-dinitrophenylhydrazine with pyrroloquinoline quinone in porcine kidney diamine oxidase. FEBS Lett. **206**:111–114.

20a. **van Kleef, M. A. G., and J. A. Duine.** 1988. L-Tyrosine is the precursor of PQQ biosynthesis in *Hyphomicrobium* X. FEBS Lett. **237**:91–97.

21. **van Schie, B. J., K. J. Hellingwerf, J. P. van Dijken, M. G. L. Elferink, J. M. van Dijl, J. G. Kuenen, and W. N. Konings.** 1985. Energy transduction by electron transfer via a pyrroloquinoline-quinone-dependent glucose dehydrogenase in *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus*. J. Bacteriol. **163**:493–499.
22. **van Schie, B. J., J. P. van Dijken, and J. G. Kuenen.** 1984. Non-coordinated synthesis of glucose dehydrogenase and its prosthetic group in *Acinetobacter* and *Pseudomonas* species. FEMS Microbiol. Lett. **24**:133–138.