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Identification of *Escherichia coli ubiB*, a Gene Required for the First Monooxygenase Step in Ubiquinone Biosynthesis

WAYNE W. POON,[1] DIANA E. DAVIS,[1] HUAN T. HA,[1] TANYA JONASSEN,[1]
PHILIP N. RATHER,[2] AND CATHERINE F. CLARKE[1]*

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90095,[1] and Departments of Medicine and Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106[2]

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It was recently discovered that the *aarF* gene in *Providencia stuartii* is required for coenzyme Q (CoQ) biosynthesis. Here we report that *yigR*, the *Escherichia coli* homologue of *aarF*, is *ubiB*, a gene required for the first monooxygenase step in CoQ biosynthesis. Both the *P. stuartii aarF* and *E. coli ubiB* (*yigR*) disruption mutant strains lack CoQ and accumulate octaprenylphenol. Octaprenylphenol is the CoQ biosynthetic intermediate found to accumulate in the *E. coli* strain AN59, which contains the *ubiB409* mutant allele. Analysis of the mutation in the *E. coli* strain AN59 reveals no mutations within the *ubiB* gene, but instead shows the presence of an IS*1* element at position +516 of the *ubiE* gene. The *ubiE* gene encodes a *C*-methyltransferase required for the synthesis of both CoQ and menaquinone, and it is the 5′ gene in an operon containing *ubiE*, *yigP*, and *ubiB*. The data indicate that octaprenylphenol accumulates in AN59 as a result of a polar effect of the *ubiE*::IS*1* mutation on the downstream *ubiB* gene. AN59 is complemented by a DNA segment containing the contiguous *ubiE*, *yigP*, and *ubiB* genes. Although transformation of AN59 with a DNA segment containing the *ubiB* coding region fails to restore CoQ biosynthesis, transformation with the *ubiE* coding region results in a low-frequency but significant rescue attributed to homologous recombination. In addition, the *fre* gene, previously considered to correspond to *ubiB*, was found not to be involved in CoQ biosynthesis. The *ubiB* gene is a member of a predicted protein kinase family of which the *Saccharomyces cerevisiae ABC1* gene is the prototypic member. The possible protein kinase function of UbiB and Abc1 and the role these polypeptides may play in CoQ biosynthesis are discussed.

Ubiquinone (coenzyme Q, or CoQ) is a prenylated benzoquinone that functions in the respiratory electron transport chain of the inner mitochondrial membranes of eukaryotes and in the plasma membrane of prokaryotes (7). In addition to respiratory electron and proton transport, the redox properties of CoQ allow the reduced form ($CoQH_2$) to scavenge lipid peroxyl radicals either directly or indirectly as mediated through α-tocopherol (6, 16, 23). This antioxidant function of $CoQH_2$ serves to protect cells from the oxidative, damaging effect of polyunsaturated fatty acids (15). Although CoQ is found primarily in the mitochondria of eukaryotes, it is also found in other organelles and in the plasma membrane, where it participates in a plasma membrane electron transport system (reviewed in reference 41). In the plasma membrane of prokaryotes, CoQ also functions in disulfide bond formation of periplasmic proteins (3, 27).

Generally, cells rely on de novo synthesis for their source of CoQ. Numerous studies carried out with bacteria and yeast have enabled the pathway to be elucidated (for reviews, see references 21, 32, and 38). Briefly, CoQ biosynthesis begins with the formation of compound 1 (Fig. 1) from the precursors polyprenyldiphosphate and 4-hydroxybenzoic acid, followed by a series of ring modifications. In *Escherichia coli*, prenylation is followed by decarboxylation, hydroxylation, and methylation, whereas in *Saccharomyces cerevisiae*, the order is hydroxylation, methylation, and then decarboxylation. The pathways then converge at compound 6 in both eukaryotes and prokaryotes. In *E. coli*, the tail of CoQ has eight isoprene groups and hence is designated CoQ_8.

A number of studies have recently focused on the methylation steps in CoQ biosynthesis. The *ubiE* gene, encoding the *E. coli C*-methyltransferase enzyme, was identified and found to be necessary for both CoQ_8 and menaquinone-8 (MK_8) biosynthesis (28). The corresponding *C*-methyltransferase in yeast was identified as *COQ5* (4, 14). One *O*-methyltransferase, identified as UbiG in *E. coli* and Coq3 in yeast, catalyzes both O-methylation steps in CoQ biosynthesis (36). However, the gene-enzyme relationships involving the monooxygenase steps in CoQ biosynthesis are not well understood. Early studies of bacteria identified mutants blocked in each of the three monooxygenase steps of CoQ_8 synthesis, and the affected genes were designated *ubiB* (12) *ubiF* (43), and *ubiH* (44). Only the *ubiH* gene has been isolated (33). Studies of yeast have identified a mitochondrial protein, Coq7p, that is necessary for the last monooxygenase step (20, 31). Homologues of Coq7 in rat, human, and the nematode *Caenorhabditis elegans* each function to restore biosynthesis of CoQ in the yeast *coq7*Δ mutant (17, 19, 40). However, the *COQ7* gene does not contain any homology to *ubiH* or any other known monooxygenase or hydroxylase. There is no known homologue for this gene in *E. coli*, but it is present in *Rickettsia* (2). Interestingly, the *C. elegans COQ7* homologue, *clk-1*, has been implicated in a life extension phenotype (17). Thus, the exact function of Coq7 in CoQ biosynthesis and its numerous pleiotropic effects remain largely unknown. The genes responsible for the other monooxygenase steps in eukaryotes have yet to be identified.

The analysis of the *E. coli* genome by Daniels et al. (13) suggested that the *fre* gene, coding for NAD(P)H flavin oxi-

* Corresponding author. Mailing address: Department of Chemistry and Biochemistry, University of California, Los Angeles, 607 Charles E. Young Dr. East, Los Angeles, CA 90095-1569. Phone: (310) 825-0771. Fax: (310) 206-5213. E-mail: cathy@mbi.ucla.edu.

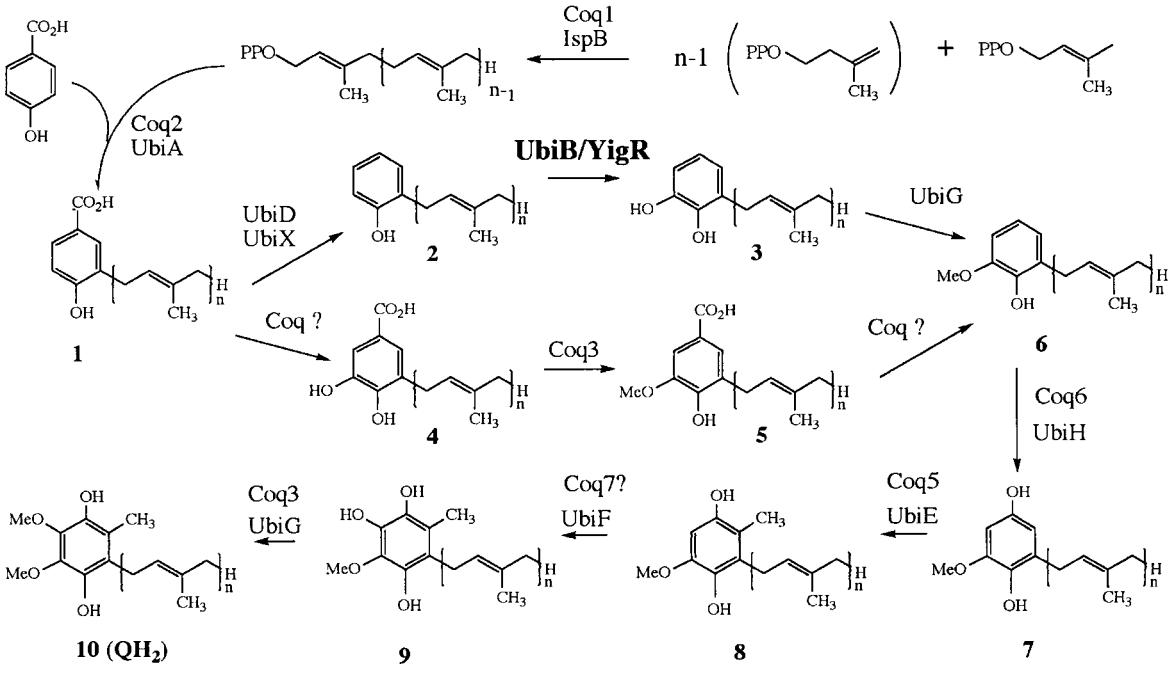

FIG. 1. Biosynthesis of CoQ in prokaryotes and eukaryotes. After formation of 3-polyprenyl-4-hydroxybenzoic acid (compound 1), the proposed biosynthetic pathways for CoQ in eukaryotes and in prokaryotes are thought to diverge. The intermediates in the pathway are 2-polyprenylphenol (compound 2); 2-polyprenyl-6-hydroxy-phenol (compound 3); 3,4-dihydroxy-5-polyprenylbenzoic acid (compound 4); 3-methoxy-4-hydroxy-5-polyprenylbenzoic acid (compound 5); 2-polyprenyl-6-methoxy-phenol (compound 6); 2-polyprenyl-6-methoxy-1,4-benzoquinol (compound 7); 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol or 5-demethoxyubiquinol (compound 8); 2-polyprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol or demethyl-QH_2 (compound 9); and CoQ_nH_2 (compound 10). In *E. coli*, n = 8, and compound 2 is referred to as octaprenylphenol. *E. coli* gene products are identified as Ubi; *S. cerevisiae* gene products are identified as Coq.

doreductase, corresponds to *ubiB* based on its chromosome location and its sequence similarity to flavin-dependent monooxygenases. *E. coli ubiB* mutants lack CoQ_8 and accumulate the Q-intermediate octaprenylphenol (compound 2, Fig. 1) (12). However, in this study, we have found that *E. coli fre* disruption mutants continue to produce CoQ, thereby excluding *fre* as a candidate for *ubiB*.

Recent work with the opportunistic pathogen *Providencia stuartii* identified *aarF* as a gene required for CoQ biosynthesis (30). The *E. coli* homologue of *aarF* was identified as *yigR*, and the *E. coli* strain DM123 harboring a disruption in the *yigR* gene was also found to be CoQ deficient (30). Both the *P. stuartii aarF* and *E. coli yigR* mutant strains accumulate compound 2, the same CoQ biosynthetic intermediate found to accumulate in AN59, a *ubiB* mutant strain of *E. coli*. In this work, we show that *aarF* in *P. stuartii* and *yigR* in *E. coli* both correspond to *ubiB*. Surprisingly, AN59 was found to contain an insertion element (IS*1*) located within the *ubiE* gene. The data presented here indicate that the IS*1* element impairs the transcription from *ubiE* and from *ubiB* (*yigR*), which corresponds to the third gene in an operon containing *ubiE*, *yigP*, and *ubiB* (*yigR*). The data presented here identify *yigR* and *aarF* as *ubiB*, a gene required for the first monooxygenase step in CoQ biosynthesis in prokaryotes.

MATERIALS AND METHODS

Strains and growth media. The *E. coli* and *P. stuartii* strains and the plasmids used in this study are listed in Table 1. Sequence analysis (GenBank accession no. 2367309) of the DNA segment containing the *ubiE-yigPQR* region indicates that the *E. coli yigQ* and *yigR* genes actually correspond to one contiguous coding region, now referred to as *ubiB*. Wild-type and mutant strains were grown in Luria-Bertani (LB) broth overnight at 37°C with vigorous shaking (350 rpm) unless otherwise indicated. Strains harboring plasmids were grown in media supplemented with ampicillin (100 μg/ml). *E. coli* strain AN59 was grown in brain heart infusion media (Difco), and gentamicin was included in the growth media to select against revertants (2 μg/ml in liquid media; 10 μg/ml in plate media). Succinate defined medium was prepared according to the method of Poole et al. (34). The transformation of strains by electroporation was carried out with the Bio-Rad gene pulser apparatus using 0.1-cm cuvettes as described by Bio-Rad.

Lipid extraction. Aliquots (5 ml) of overnight cultures were used to inoculate 1 liter of the corresponding media, containing 0.45 μCi of *p*-[U-^{14}C]hydroxybenzoic acid per liter. The synthesis of *p*-[U-^{14}C]hydroxybenzoic acid (364.8 Ci/mol, 25.2 μCi/ml of ethanol) has been described previously (35). Cultures were harvested (16 to 20 h) and subjected to lipid extraction as described previously (35, 37). The organic layer was reduced to approximately 30 ml by a stream of nitrogen. The extracts were washed thrice (1 ml each time) with distilled water. The samples were then completely evaporated under a stream of N_2, and the lipids were resuspended in 0.5 ml of hexane per liter of original culture.

Purification of CoQ_8, MK_8, and intermediates. Lipid samples were either subjected to SepPak chromatography or directly analyzed by high-pressure liquid chromatography (HPLC). C_{18} SepPak cartridges were obtained from Millipore Corporation (Bedford, Mass.). The column was equilibrated with 7 ml of acetonitrile, and 450 μl of extract was applied to the column. The elution solvents were described previously (18): (i) 7 ml of acetonitrile, (ii) 7 ml of acetonitrile-isopropanol (85:15), (iii) 7 ml of acetonitrile-isopropanol (7:3), (iv) 3.5 ml of isopropanol, and (v) 3.5 ml of hexane. All fractions were collected, dried down completely, and then resuspended in 300 μl of hexane. Each fraction was monitored for radioactivity by scintillation counting. Fraction 2 contained greater than 60% of the radioactivity. This fraction was evaporated under a stream of N_2 and was resuspended in hexane prior to separation by HPLC. For the samples of *E. coli* and *P. stuartii* lipid extracts, the elution profiles of radioactivity were very similar in the total lipid extract and in fraction ii.

The Gilson HPLC system was composed of two 306 pumps, an 811C mixing chamber, a 118 UV-VIS spectrophotometer, a 506C system interface, and a model 203B fraction collector. Data were collected with Gilson 715 system software. The reverse-phase column (Econosphere C_{18}, 5 μm, 4.6 by 250 mm; Alltech) was equilibrated for 1 h (flow rate of 1 ml/min) in acetonitrile-isopropanol (75:25), and upon sample injection, the percentage of isopropanol was increased to a final ratio of 40:60 at 35 min. An aliquot of hexane ("sample blank") was injected prior to analysis of the lipid extracts. The radioactivity in each 1-ml sample was determined by scintillation counting. Alternatively, if a sample was needed for mass spectrometry, 200 μl of each HPLC fraction was subjected to scintillation counting, and radioactive fractions were evaporated under a stream of nitrogen, resuspended in 25 μl of hexane, and analyzed by high-resolution (electron impact) mass spectrometry.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
E. coli strains		
HW272	*ubiG*$^+$ *zei*::Tn*10*dTet	42
GD1	*ubiG*::Kan *zei*::Tn*10*dTet	18
RM1734	(MG1655)1$^-$ F$^-$ *rph*-1 (wild type)	30
DM123	RM1734 *yigR*::Kan	30
AN59	Hfr, *thr-1 leu-6 ubiE409*::IS*1*	12[a]
LS1312	*fre*::Kan	DiRusso[b]; 11
P. stuartii strains		
PR50	Wild type	30
PR54	PR50 *aarF*::Cm	30
Plasmids		
pET21a	T7 expression plasmid, Ap	Novagen
pEF1	pET21a::3.5-kb *Sau*3A1 fragment containing *E. coli ubiE* and *yigP* and *ubiB* genes	30
pSK-2.6	pBluescript SK(−)::2.6-kb *Sal*I fragment from pEF1 (*yigP* and *ubiB* genes)	30
pSK-aarF	pBluescript SK(−)::1.9-kb fragment from pAFM12 (*P. stuartii aarF*)	30
pQM	Vector containing the yeast *CYC1* promoter	18
pUE-3	pQM::*ubiE* coding region	28

[a] The mutant allele in this strain has been characterized as *ubiE409*::IS*1* and replaces the original designation "*ubiB409*."
[b] Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, N.Y.

Determination of CoQ levels. Levels of CoQ were determined as described before (22) except that bacteria were grown overnight in LB broth (DH5α) or LB broth plus kanamycin (LS1312) or were grown for 3 days to stationary phase (DM123, HW272, and GD1).

PCR amplification, plasmid construction, and DNA sequence analysis. Segments of genomic DNA from lysates of DH5α and LS1312 were amplified using multiple combinations of the primers FRE1 (5′-GTGACCTCGGTAGAAGCT ATCACGGAT-3′), FRE2 (5′-GCTGGTCAGTATTTGATGGTAGTGATG-3′), FRE3 (5′-TCCGGCAATATAGATATCATGCTCTGC-3′), and FRE4 (5′-ACGCTCACTGCAAAACAGATCGCGGGC-3′). The combinations of FRE1 and FRE3, FRE2 and FRE3, and FRE2 and FRE4 in the wild-type strain resulted in PCR products with sizes of 583 nucleotides (nt), 511 nt, and 558 nt, respectively. However, all of these products were approximately 1 kb larger in LS1312, verifying the presence of the kanamycin cassette. DNA sequence analysis of the *ubiE* open reading frame (ORF) in the mutant strain AN59 was determined by dideoxynucleotide chain termination with a Sequenase kit (U.S. Biochemicals) and α-^{35}S-dATP (1,069 Ci/mmol; NEN Research Products). The DNA was sequenced from a PCR-amplified product of AN59 genomic DNA with the following primers for amplification: pAN70-1 (5′-TTCATCGATGAC ATGTCCGC-3′, from 71559 to 71578, corresponding to the site 350 bp upstream of the *ubiE* ATG codon) and pAN70-2 (5′-AATACTTTACCCAGCAGACG-3′, from 72806 to 72787, corresponding to the site 104 bp downstream of the *ubiE* stop codon). The PCR fragment was gel purified and inserted via blunt-end ligation into the end-filled *Bam*HI site of pBluescript (Stratagene). T3 and T7 primers were used in the initial sequencing reactions; subsequent primers were made based on the sequences obtained from the previous primers, and the amplified segment was sequenced in both directions. The *ubiE* ORF was sequenced completely, while the insertion element was 90% sequenced.

RESULTS

An *E. coli fre* disruption mutant continues to produce CoQ_8. The *E. coli fre* gene, encoding NAD(P)H flavin oxidoreductase (11), had been tentatively identified as *ubiB* based on its map position and its sequence similarity to subunits of other prokaryotic monooxygenases (13, 32). The involvement of the *fre* gene in CoQ biosynthesis was investigated in the mutant strain LS1312 containing a disruption in the *fre* gene (11). The presence of the *fre*::Kan disruption allele in LS1312 was confirmed by PCR (see Materials and Methods), and total lipid extracts were prepared from this strain and analyzed for the amount of CoQ_8 by HPLC and electrochemical detection as described previously (22). The level of CoQ_8 in LS1312 (32.6 ± 3.6 ng of Q_8 per mg [dry weight] of cells) was not significantly different from that present in a laboratory wild-type strain of *E. coli* DH5α (40.3 ± 6.2 ng of CoQ_8 per mg [dry weight] of cells). The presence of CoQ_8 in LS1312, combined with normal growth on succinate defined medium, excludes the *fre* gene as a candidate gene for *ubiB*.

Accumulation of octaprenylphenol, a CoQ precursor, in *aarF* or *yigR* (*ubiB*) mutants. To identify the CoQ intermediates accumulating in the recently identified CoQ-deficient mutant strains of *P. stuartii* and *E. coli* (30), growth media were supplemented with *p*-[U-^{14}C]hydroxybenzoic acid, and lipid extracts were prepared and separated by HPLC as described in Materials and Methods. The UV profiles for the lipid extracts from the wild-type strains demonstrated a major peak eluting at 17 to 18 min and coeluted with a CoQ_8 standard (Fig. 2A and G). The subsequent analysis of incorporated radioactivity revealed a large amount of radiolabel that coincided with the UV peak in fraction 18 (Fig. 2D and J). Electron impact mass spectral analysis of fraction 18 from both wild-type strains gave molecular ions which correspond to CoQ_8 (M = $C_{49}H_{74}O_4$: 726.558712; Fig. 2D observed mass, 726.556882; ppm, 2.5; Fig. 2J observed mass, 726.560768; ppm, −2.8). Since the separation of the *E. coli* wild-type lipid extract produced two major radioactive fractions, fraction 16 (Fig. 2J) was also analyzed, and the molecular ion corresponds to CoQ_8H_2 (M = $C_{49}H_{76}O_4$: 728.574362; observed mass, 728.573189; ppm, 1.6).

Both *P. stuartii aarF* and *E. coli yigR* mutants (PR54 and DM123, respectively) had lipid profiles that were distinct from wild type, since the large UV peak associated with CoQ_8 was absent, and instead a large UV peak eluted at 20 to 21 min (Fig. 2B and H). The radioactivity profile was also different for the mutants, since no radioactivity was detected in the position expected for CoQ_8 at fraction 18 (Fig. 2E and K). Analysis of this fraction by mass spectrometry found no molecular ions for either CoQ_8 or CoQ_8H_2. Instead, these mutants accumulated radiolabeled material that eluted at 20 to 21 min, coinciding with the major UV peak (Fig. 2B and H). Electron impact mass spectral analysis of the radiolabeled product in fraction 20 indicated that it was the CoQ precursor, octaprenylphenol (compound 2) (M = $C_{46}H_{70}O$: 638.542667; Fig. 2E observed mass, 638.544334; ppm, −2.6; and Fig. 2K observed mass, 638.540401; ppm, 3.5). Transformation of PR54 and DM123 with DNA clones containing either *aarF* or *yigR* genes (pSK-aarF and pSK-2.6, respectively) restored the production of CoQ_8 as determined by HPLC and mass spectral analysis (fraction 18; Fig. 2F observed mass, 726.557703; ppm, 1.4; and Fig. 2L observed mass, 726.557122; ppm, 2.2). The rescue of the mutants harboring the *aarF*::Cm or *ubiB*::Kan disruption alleles was inefficient, as high levels of octaprenylphenol were

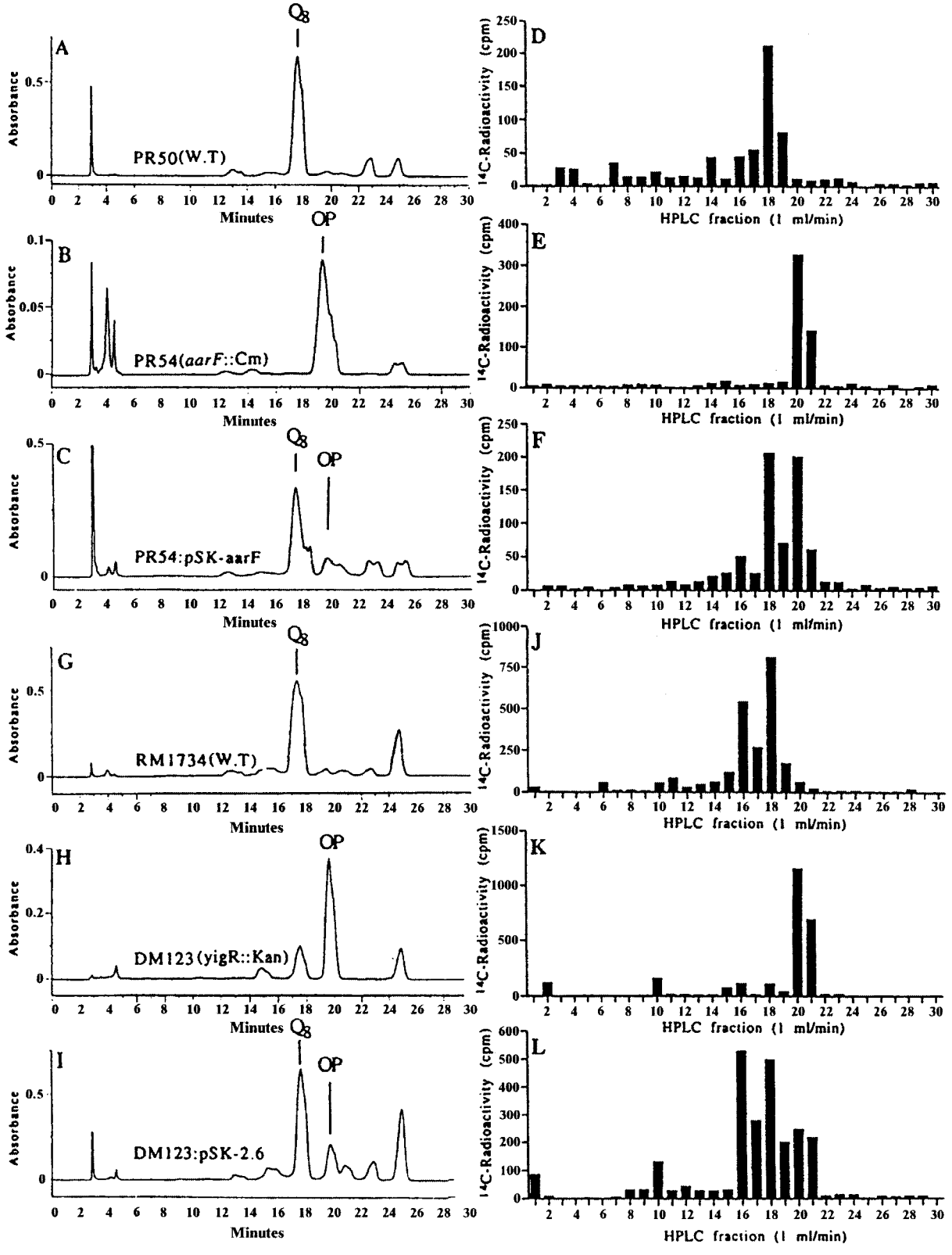

FIG. 2. Disruption of the *aarF* gene in *P. stuartii* or the *yigR* gene in *E. coli* blocks production of CoQ_8 and leads to the accumulation of octaprenylphenol (compound 2). Lipid extracts from *P. stuartii* (A to F) or *E. coli* (G to L) strains labeled with *p*-[U-^{14}C]hydroxybenzoic acid were separated by reverse-phase HPLC. Absorbance (272 nm) is depicted in A to C and G to I, and ^{14}C radioactivity present in each fraction is depicted in D to F and J to L. The profiles of absorbance and radioactivity correspond to (i) designated strains of *P. stuartii*, namely, PR50, wild-type (A and D); PR54, *aarF* mutant (B and E); or PR54 rescued with the pSK-aarF plasmid (C and F); and (ii) the *E. coli* strain RM1734 (G and J), DM123 (H and K), or DM123/pSK-2.6 (I and L).

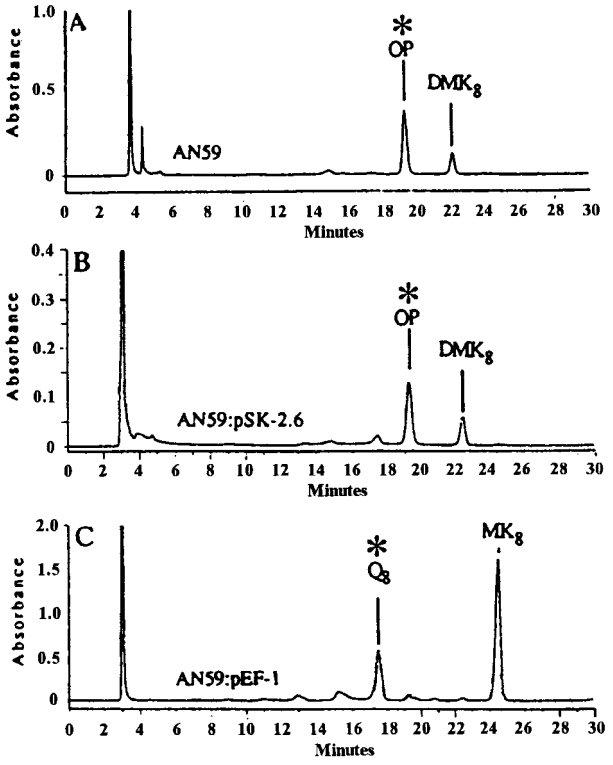

FIG. 3. AN59 lacks CoQ_8 and accumulates octaprenylphenol and DMK_8. Lipid extracts prepared from *E. coli* strains labeled with *p*-[U-^{14}C]hydroxybenzoic acid were separated by reverse-phase HPLC as described in Materials and Methods. Absorbance (272 nm) is depicted. The major peak of radioactivity for each strain analyzed by mass spectroscopy is depicted (*). The profiles of absorbance correspond to AN59 (A), AN59/pSK-2.6 (B), and AN59/pEF1 (C). OP, octaprenylphenol.

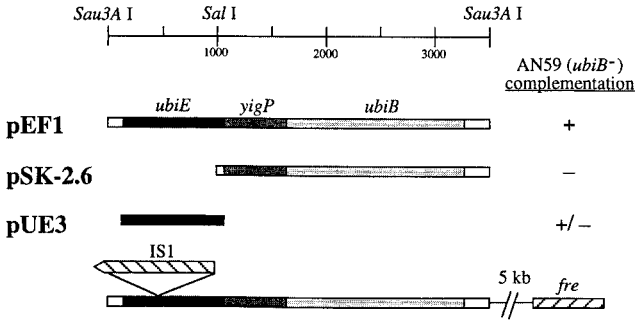

FIG. 4. Identification of the mutation in AN59 (*ubiE409*::IS*1*) and determination of the sequences required for complementation. The shaded bars designate the ORFs in an operon comprised of *ubiE*, *yigP*, and *ubiB* (*yigR*). The *fre* gene is located approximately 5 kb from the 3′ end of *ubiB*. The position of a unique *Sal*I restriction site is shown; the two *Sau*3A sites represent the ends of the fragment which was generated by partial digestion (30). Complementation of the slow-growth phenotype by plasmids derived from pEF1 is shown. +, restoration of wild-type growth rate and rescue of growth on succinate minimal media; –, failure to restore wild-type growth rate and growth on succinate minimal media; ±, mixed population with a preponderance of small, slow-growth colonies.

still detected (see Fig. 2F and L). The restoration of CoQ_8 biosynthesis is consistent with the finding that plasmids containing the *aarF* or *yigR* genes restored the ability of the mutant strains (PR54 and DM123, respectively) to utilize succinate as the sole carbon source (30). The identification of octaprenylphenol as the accumulating intermediate in DM123 indicated a deficiency in the biosynthetic step mediated by the *ubiB* gene product (12). The rescue of this defect by the *yigR* gene product suggests that *yigR* corresponds to *ubiB*.

Accumulation of both octaprenylphenol and demethylmenaquinone in AN59. Work by Cox et al. (12) characterized the *E. coli* mutant strain AN59 as accumulating octaprenylphenol (compound 2). Our analysis of the *p*-[U-^{14}C]hydroxybenzoic acid-radiolabeled lipid extracts from AN59 confirmed this finding. Electron impact mass spectral analysis of radioactive fractions for AN59 (denoted by the asterisk in Fig. 3A) identified the CoQ-intermediate octaprenylphenol (M = $C_{46}H_{70}O$: 638.542667; observed mass, 638.543099; ppm, −0.7). In the same lipid extract, significant amounts of UV-absorbing material eluted at 22 min but lacked radioactivity. Mass spectral analysis of this UV peak revealed the presence of demethylmenaquinone-8 (DMK_8; M = $C_{50}H_{70}O_2$: 702.537582; observed mass, 702.537439; ppm, 0.2). The presence of DMK_8 in the AN59 mutant strain suggests a defect in the *ubiE* gene, since the accumulation of DMK_8 was previously observed in the *ubiE* mutant strain AN70 (28). The presence of a large UV-absorbing peak but a lack of radioactivity is expected here since MK_8, unlike CoQ_8, is not derived from the CoQ precursor *p*-hydroxybenzoic acid.

IS*1* insertion mutation in the *ubiE* gene of AN59. The results of the lipid analysis described above suggested that the mutation in AN59 may lie within the *ubiE* gene. PCR amplification of the *ubiE* region from genomic DNA of AN59 consistently produced a single DNA fragment of approximately 2,000 bp, 800 bp larger than the product observed with wild-type genomic DNA. Sequencing of this fragment revealed the presence of IS*1* in an inverse orientation at nt 516 (Fig. 4). The presence of this element explains the high rate of reversion observed for AN59 by the ability of insertion sequences to self-excise from the DNA. It follows that the accumulation of octaprenylphenol in AN59 results from polar effects of the IS*1* mutation on the downstream *ubiB* gene in the *ubiE* operon.

Determination of the sequences required for complementation of AN59. The accumulation of octaprenylphenol in both AN59 (*ubiB* mutant) and DM123 (*ubiB*::Kan) suggested that the locus may correspond to *ubiB*. To examine this, the plasmids shown in Fig. 4 were introduced into AN59, and the resulting transformants were tested for growth on succinate defined media. Transformation of AN59 with pEF1 rescued the slow growth phenotype of AN59 and restored growth on media containing succinate. Transformation with pSK-2.6 generated only small "pinpoint" colonies that do not utilize succinate. However, transformation of AN59 with pUE-3 generated a mixed population of colonies: a majority of small pinpoint colonies and a minority of large wild-type-size colonies. Transfer of the small colonies by restreaking to LB plate media again resulted in a mixed population containing mostly small colonies but with large colonies appearing at a high frequency. These complementation results suggest that the IS*1* element is removed by homologous recombination within the *ubiE* ORF and that such repair produces the large colony (succinate$^+$) phenotype.

Lipid extracts were prepared from AN59 harboring either pEF1 or pSK-2.6 to examine the effects of these plasmids on the synthesis of CoQ_8 and MK_8. The major radioactive fractions corresponded to the UV peaks labeled in Fig. 3. Mass spectral analysis of radiolabeled lipids from AN59 transformed with pEF1 demonstrated the presence of CoQ_8 (M = $C_{49}H_{74}O_4$: 726.558712; Fig. 3C observed mass, 726.555928;

ppm, 3.8). In addition, the lipid analysis of AN59 transformed with pEF1 demonstrated the presence of a UV peak in fraction 25 (Fig. 3C) that corresponded to MK_8 (M = $C_{51}H_{72}O_2$: 716.553232; Fig. 3C observed mass, 716.552534; ppm, 1.0). Thus, the presence of the pEF1 restores the synthesis of both CoQ_8 and MK_8. Analysis of the radiolabeled lipids from AN59/pSK-2.6 showed a large radioactive peak at fraction 20 corresponding to the UV peak (Fig. 3B) and was identified as octaprenylphenol (M = $C_{46}H_{70}O$: 638.542667; observed mass, 638.544103; ppm, −2.2). No other radioactive peak was detected. However, a UV peak was detected in fraction 23 (Fig. 3B) which corresponded to DMK_8 when analyzed by mass spectrometry (M = $C_{50}H_{70}O_2$: 702.537582; Fig. 3E observed mass, 702.537407; ppm, 0.2).

***ubiB* Disruption mutant DM123 produces low levels of CoQ_8 in stationary phase.** It was noted that lipid extracts prepared from DM123 grown to stationary phase contained significant levels of CoQ_8 (48.7 ± 2.8 ng of CoQ_8 per mg [dry weight] of cells). These levels were lower than those detected in stationary-phase cultures of the wild-type strain HW272 (184.0 ± 5.0 ng of CoQ_8 per mg [dry weight] of cells). The production of CoQ_8 in DM123 stationary-phase cultures was not accounted for by reversion or suppression mutations because aliquots of the culture were still unable to grow when transferred to succinate plate media. Previous studies on mutant strains defective in one of the monooxygenase steps (*ubiB*, *ubiH*, or *ubiF*) showed that each defect in CoQ_8 biosynthesis was bypassed under anaerobic growth conditions (1). *E. coli ubi* mutants that are blocked at other steps of CoQ biosynthesis (e.g., *ubiD*, *ubiE*, and *ubiA*) do not exhibit a bypass in response to anaerobic culture conditions (1). In the *ubiB*, *ubiH*, and *ubiF* strains, alternate hydroxylases appear to be responsible for anaerobic CoQ_8 biosynthesis. It seems likely that this type of bypass is operating under stationary-phase growth conditions of our experiments. Furthermore, the production of CoQ_8 at stationary phase was specific for the *ubiB* defect, since CoQ_8 biosynthesis was not detected in stationary-phase cultures of the *ubiG* deletion strain GD1. Hence, the role of *ubiB* in the first monooxygenase step in CoQ biosynthesis is specific for aerobically grown log-phase cells, and CoQ synthesis can occur in stationary-phase cells despite the defect in *ubiB*.

DISCUSSION

The results presented provide strong evidence for the identification of the *ubiB* gene in *E. coli*. Disruption mutants of *ubiB* in *E. coli* and *ubiB* (*aarF*) in *P. stuartii* each accumulate octaprenylphenol, the sole CoQ intermediate expected to accumulate in *E. coli* blocked at the first monooxygenase step. The nature of the CoQ biosynthetic defect in AN59 (the original *ubiB* mutant) was discovered to be an IS*1* element located in the *ubiE* coding region causing a polar mutation that affects the downstream *ubiB* gene and results in the accumulation of octaprenylphenol.

Lipid analyses of AN59/pSK-2.6 transformants contain the intermediates octaprenylphenol (compound 2 in Fig. 1) and DMK_8. This is surprising since we predicted that AN59/pSK-2.6 would produce compound 7, the same intermediate that is observed in *ubiE* mutant strains (28). We speculate that the rescue provided by the *aarF* or *ubiB* plasmid constructs may not be as efficient. This could be due to overexpression resulting in aggregated or inactive protein, or possibly the promoter driving expression of *ubiB* or *aarF* is suboptimal relative to the normal chromosomal promoter. This hypothesis also explains the high levels of octaprenylphenol detected in the PR54 and DM123 mutants (harboring the *aarF*::Cm or *ubiB*::Kan disruption alleles, respectively) that were rescued with plasmids carrying the wild-type gene. In our previous experience with the *ubiE* and *ubiG* mutants, rescue by the corresponding wild-type genes was associated with the disappearance of the characteristic CoQ intermediate peak.

The functional role of the *ubiB* gene product in the hydroxylation of octaprenylphenol is unknown. However, the presence of a homologue in *P. stuartii* demonstrates the conservation of function found in nature for this gene product. The *ubiB* gene does not contain sequence identity corresponding to known monooxygenases in the databases. Examination of *ubiB* shows that it shares sequence identity with the *ABC1* gene in *S. cerevisiae*, which is required for function of the mitochondrial bc_1 complex (5, 8), in which CoQ functions as an essential cofactor. Recently, the *Arabidopsis thaliana* homologue of *ABC1* was identified through functional complementation of a yeast *ABC1* deletion mutant (9). Thus, the function of *ABC1* in the biosynthesis of CoQ is likely to be conserved. Cardazzo et al. (9) have shown that Abc1 and Abc1-related proteins are part of a large family of proteins in both eukaryotes and prokaryotes, including *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Clostridium pasteurianum*. The presence of *ABC1* in *M. tuberculosis* and *M. leprae* is curious since these gram-positive bacteria are generally considered to produce menaquinone but not CoQ (10). However, inspection of the genomes of *M. tuberculosis* and *M. leprae* reveals the presence of other CoQ biosynthetic genes, including *ispB*/*COQ1*, *ubiA*, *ubiG*, *ubiE*/*COQ5*, and a candidate gene for *ubiF* (W. W. Poon, unpublished observations). Since each of these genes encodes an enzyme catalyzing a biosynthetic step of CoQ biosynthesis (see Fig. 1 and references 21, 32, and 38), it seems likely that both *M. tuberculosis* and *M. leprae* do in fact produce CoQ. The presence of a *ubiB* homologue was also noted for *C. pasteurianum* (9). This gram-positive unicellular endospore-forming prokaryote is considered to lack both isoprenylated quinones and cytochromes (10). It is not currently possible to determine whether other CoQ biosynthetic genes are present in *C. pasteurianum*, as the complete genome sequence is not available. For this organism, the putative kinase function of the protein may have been conserved but its role in CoQ synthesis is questionable. It is possible that the homologue in *C. pasteurianum* corresponds to one of the *ABC1*-like homologues that are not required for respiration (9).

Along with *ABC1*, *ubiB* is part of a large family of proteins that contain motifs found in eukaryotic-type protein kinases (29). Although it is not known if the protein encoded by *ubiB* contains kinase activity or what substrates it may act on, it is interesting to speculate that UbiB may play a role in ubiquinone biosynthesis-activating proteins necessary for the monooxygenase step(s) via phosphorylation. Previous work had identified a pool of octaprenylphenol that remained bound to a protein complex until a signal such as oxygen activated the complex to continue the ring modifications required in CoQ biosynthesis (24, 25). The rapid conversion of octaprenylphenol to CoQ_8 (26) upon transfer from anaerobic to aerobic growth conditions is consistent with a mechanism requiring a kinase(s) to regulate such a response. This may also be the function of the yeast homologue *ABC1*, since recent analyses of yeast *abc1* mutants show that the *ABC1* gene is required for CoQ biosynthesis (T. Q. Do and C. F. Clarke, unpublished data).

The possibility of regulation of CoQ biosynthesis by phosphorylation is intriguing. The synthesis of CoQ, an essential component of the electron transport chain, would constitute a potential site for kinase regulation of energy metabolism. There is ample precedent for the involvement of kinases in

energy metabolism (e.g., phosphorylation control of glycogen and fatty acid metabolism) (39). In *E. coli* mutants that are defective in one of the monooxygenase steps, CoQ is synthesized in the absence of oxygen, suggesting that an alternative hydroxylase functions under anaerobic conditions (1). These questions arise: how is CoQ synthesis regulated in aerobic versus anaerobic cultures, and does *ubiB* play a role in such regulation? Furthermore, is this mechanism also present when *E. coli* shift from early log phase to stationary-phase growth conditions? The data presented here show that CoQ is synthesized differently in log phase and stationary phase, since the defect in *ubiB* seems to affect only CoQ biosynthesis in log-phase cultures and is bypassed in stationary phase, similar to the bypass mechanism found in anaerobically grown cells. This bypass could be the result of differential phosphorylation by the *ubiB* gene product during growth, and therefore it will be important to investigate the possibility of *ubiB* kinase activity. Additional studies on *ubiB* and its exact role in CoQ biosynthesis will further examine this scenario.

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