Complementation of *coq3* Mutant Yeast by Mitochondrial Targeting of the *Escherichia coli* UbiG Polypeptide: Evidence That UbiG Catalyzes Both *O*-Methylation Steps in Ubiquinone Biosynthesis†

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ABSTRACT: Ubiquinone functions in the mitochondrial electron transport chain. Recent evidence suggests that the reduced form of ubiquinone (ubiquinol) may also function as a lipid soluble antioxidant. The biosynthesis of ubiquinone requires two *O*-methylation steps. In eukaryotes, the first *O*-methylation step is carried out by the Coq3 polypeptide, which catalyzes the transfer of a methyl group from *S*-adenosylmethionine to 3,4-dihydroxy-5-polyprenylbenzoate. In *Escherichia coli*, 2-polyprenyl-6-hydroxyphenol is the predicted substrate; however, the corresponding *O*-methyltransferase has not been identified. The second *O*-methylation step in *E. coli*, the conversion of demethylubiquinone to ubiquinone, is carried out by the UbiG methyltransferase, which is 40% identical in amino acid sequence with the yeast Coq3 methyltransferase. On the basis of the chemical similarity of the first and last methyl-acceptor substrates and the high degree of amino acid sequence identity between Coq3p and UbiG, the ability of UbiG to catalyze both *O*-methylation steps was investigated. The current study shows that the *ubiG* gene is able to restore respiration in the yeast *coq3* mutant, provided *ubiG* is modified to contain a mitochondrial leader sequence. The mitochondrial targeting of *O*-methyltransferase activity is an essential feature of the ability to restore respiration and hence ubiquinone biosynthesis *in vivo*. *In vitro* import assays show the mitochondrial leader sequence present on Coq3p functions to direct mitochondrial import of Coq3p *in vitro* and that processing to the mature form requires a membrane potential. *In vitro* methyltransferase assays with *E. coli* cell lysates and synthetically prepared farnesylated-substrate analogs indicate that UbiG methylates both the derivative of the eukaryotic intermediate, 3,4-dihydroxy-5-farnesylbenzoate, as well as that of the *E. coli* intermediate, 2-farnesyl-6-hydroxyphenol. The data presented indicate that the yeast Coq3 polypeptide is located in the mitochondria and that *E. coli* UbiG catalyzes both *O*-methylation steps in *E. coli*.

Ubiquinone (coenzyme Q or Q)[1] functions in electron and proton transport in the respiratory chain located in the inner mitochondrial membrane of eukaryotes and the plasma membrane of prokaryotes (Brandt & Trumpower, 1994). Q is also present in other intracellular membranes and is a lipid component of lipoproteins (Kalen et al., 1987; Stocker et al., 1991). Recent evidence suggests that it may serve an important alternate function as a lipid soluble antioxidant. The redox chemistry that allows Q to be reversibly reduced and oxidized in mitochondrial electron transport may also allow ubiquinol (QH_2, the hydroquinone) to scavenge lipid peroxyl radicals, a class of oxidative products that represents a major cause of damage to cellular membranes (Ernster & Dallner, 1995). There is a growing body of evidence that oxidative damage in mammalian cells is one of the major causes of cancer (Ames et al., 1995) and that oxidative modifications to low-density lipoprotein lipids may play an important role in the initiation of coronary artery disease (Navab et al., 1995).

Q/QH_2 is the only lipid soluble antioxidant that can be synthesized by mammalian cells; the other lipid soluble antioxidants (vitamin E, β-carotene) must be derived from the diet. Cells synthesize Q from the precursors *p*-hydroxybenzoate and polyisoprenediphosphate (Olson & Rudney, 1983). Figure 1 shows the proposed biosynthetic pathway as derived from the characterization of accumulating Q intermediates in Q-deficient mutant strains of *Escherichia coli* (*ubi*) and *Saccharomyces cerevisiae* (*coq*) (Olson & Rudney, 1983; Gibson, 1973). The Q mutant strains of *S. cerevisiae* fall into eight complementation groups (*coq1*−*coq8*), are non-respiring, and thus fail to grow on non-fermentable carbon sources (Tzagoloff & Dieckmann, 1990). Likewise, the *ubi* mutants of *E. coli* fail to grow on media containing succinate as the sole carbon source (Gibson, 1973). The prokaryotic pathway proceeds by initial decarboxylation of **1** followed by oxidation and *O*-methylation, while the eukaryotic pathway proceeds with the oxidation of **1** followed by *O*-methylation and decarboxylation.

There are two *O*-methylation steps required for the synthesis of Q. The first *O*-methylation step in eukaryotic cells is carried out by the *COQ3* gene product (Clarke et al., 1991; Goewert et al., 1981; Shepherd et al., 1996). The

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[1] Abbreviations: GDM, defined *E. coli* growth media containing glucose; HMPA, hexamethylphosphoric triamide; $Pd(PPh_3)_4$, tetrakis-(triphenylphosphine)palladium(0); SD-Ura, minimal yeast growth media lacking uracil; SDM, defined *E. coli* growth media containing succinate; Q, ubiquinone; QH_2, ubiquinol; YPD, rich yeast growth media with dextrose; YPG, rich yeast growth media with glycerol.

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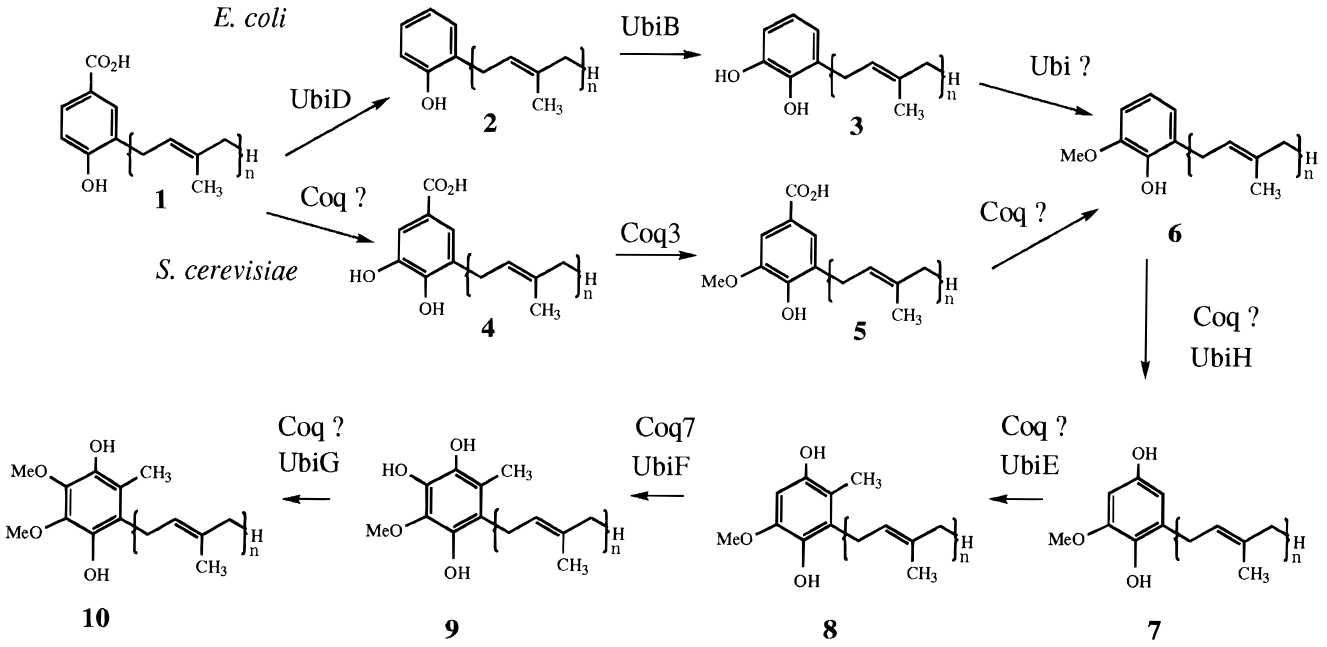

FIGURE 1: Pathway of Q biosynthesis. The proposed biosynthetic pathway for Q in eukaryotes and in prokaryotes is thought to diverge after assembly of compound **1**, 3-polyprenyl-4-hydroxybenzoate. The other intermediates in the pathway are **2**, 2-polyprenylphenol; **3**, 2-polyprenyl-6-hydroxy-phenol; **4**, 3,4-dihydroxy-5-polyprenylbenzoate; **5**, 3-methoxy-4-hydroxy-5-polyprenylbenzoate; **6**, 2-polyprenyl-6-methoxy-phenol; **7**, 2-polyprenyl-6-methoxy-1,4-benzoquinone; **8**, 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol or 5-demethoxy-ubiquinol; **9**, 2-polyprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol; and **10**, ubiquinol-*n*. Compounds **7**−**10** are drawn as hydroquinones. Compounds **6**, **7**, and **9** are hypothetical intermediates in *S. cerevisiae* Q biosynthetic pathway, as is compound **3** in *E. coli*. In *E. coli* $n = 8$; *S. cerevisiae*, $n = 6$. Gene products of different complementation groups are identified as Ubi in *E. coli* and Coq in *S. cerevisiae*.

Table 1: Genotype and Sources of *S. cerevisiae* and *E. coli* Strains

strain	genotype	source
S. cerevisiae		
JM45Δcoq3	*MAT*α, *leu2-3, leu2-112, ura3-52, trp1-289, his4-580, coq3::LEU2*	Clarke et al., 1991
JM45	*MAT*α, *leu2-3, leu2-112, ura3-52, trp1-289, his4-580*	McEwen et al., 1986
W303-1B	*MAT*α, *ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1*	Tzagoloff et al., 1975
E. coli		
HW272	*ubiG*$^+$, *zei*::Tn*10*dTet	Wu et al., 1992
HW271	*ubiG*, *zei*::Tn*10*dTet	Wu et al., 1992
AN151	*ubiG423, met*	Stroobant et al., 1972
GD1	*ubiG*::Kan, *zei*::Tn*10*dTet	this work
JC7623	*F*$^-$, *thr1, leu6, proA2, his4, thi1, argE3, lacY1, glaK2, ara14, xyl15, tsx33, rpsL31, supE44, recB21, recC22, sbcB15, sbcC201*	Kushner et al., 1971

methyltransferase responsible for the second *O*-methylation step has been partially purified from rat liver mitochondria (Houser & Olson, 1977); however, the eukaryotic gene has not yet been identified. The second *O*-methyltransferase in *E. coli* has been identified as the product of the *ubiG* gene on the basis of the characterization of the Q intermediates present in two *E. coli ubiG* mutants (Stroobant et al., 1972). Lipid analysis indicated that these *ubiG* mutants were leaky, each producing about 5% as much Q as wild-type *E. coli*. Two other intermediates, 2-octaprenylphenol (**2**) and demethyl-Q (**9**), were also present in amounts corresponding to 60% and 30% of the normal level of Q, respectively. *In vitro* assays with *E. coli* cell extracts and demethyl-Q as the methyl-acceptor substrate showed that *ubiG* mutants lacked methyltransferase activity and suggested that the *ubiG* is the structural gene encoding the second *O*-methyltransferase (Leppik et al., 1976). The first *O*-methylation (the methylation of **3** to form **6**) in *E. coli* has not been characterized. The predicted amino acid sequence of the yeast Coq3 methyltransferase is 40% identical with *E. coli* UbiG (Clarke et al., 1991; Wu et al., 1992), and both polypeptides contain consensus sequences for binding the co-substrate *S*-adenosyl-L-methionine (Kagan & Clarke, 1994). We found this degree of identity very intriguing as the first and last methyl-acceptor substrates (**4** and **9**) share similarities in structure. It seemed possible that characterization of the leaky *ubiG* mutant alleles may have masked a defect at an earlier step and that perhaps both *O*-methylation steps in Q biosynthesis might be mediated by the UbiG methyltransferase. The present study provides evidence that this scenario is indeed correct for *E. coli* UbiG.

MATERIALS AND METHODS

Strains and Growth Media. The strains of *S. cerevisiae* and *E. coli* used in this study are described in Table 1. Growth media for yeast were prepared as described (Rose et al., 1990) and included YPD (1% yeast extract, 2% peptone, 2% dextrose), YPG (1% yeast extract, 2% peptone, 3% glycerol) and SD-Ura [0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, and complete amino acid supplement lacking uracil]. Defined medium containing either succinate (SDM) or

glucose (GDM) contained 0.5% Casamino acids supplement and was prepared as described (Poole et al., 1989). The antibiotics, kanamycin and ampicillin, were both added to media as needed at final concentration of 50 μg/mL. All solid media contained 2% Difco Bacto agar. *S. cerevisiae* and *E. coli* were grown at 30 and 37 °C, respectively.

Plasmid Construction. DNA constructions were performed as described (Ausubel et al., 1995). To construct pAH01, a *CYC1* promoter DNA segment (−310 to +61) was generated by PCR from the plasmid pLGΔ-312 (Guarente & Mason, 1983) with primers pCYCE5 (5′CCGAATTCAGCAAGATCAAGATGTTTTCACCG3′) and pCYCE3 (5′CCGAATTCGTGTGTGTATTTGTGTTTGCGTGT3′) and was inserted into the *Eco*RI site of yeast CEN-based shuttle vector pRS316 (Sikorski & Hieter, 1989). DNA containing the complete *ubiG* ORF (851−1572) was generated by PCR from pRPB (Wu et al., 1992) with the primers pCC3 (5′GGAAGCTTATGAATGCCGAAAAATCGCCGGTA3′) and pCC4K (5′CCGGTACCTCACTTATTCTGCGTGTGCAGCAT3′), and the resulting 730 bp product was inserted via blunt-end ligation into the end-filled *Hin*dIII site of pAH01 to generate pAHG. DNA containing the yeast *COQ3* ORF (+1 to +951) was amplified by PCR from pRS12A-2.5Sma (Clarke et al., 1991) with the primers pCC9 (5′GGAAGCTTATGGGATTCATAATGTTGTTAAGA3′) and pCC10K (5′CCGGTACCTCAATTCAGTCTCTGAATAGCCAT3′), and the resulting 950 bp product was inserted into pAH01 as described above to generate pAH3. pAH3M is identical to pAH3 except that an *Mlu*I site in the *CYC1* promoter was removed by end-filling and re-ligation.

The first 35 amino acids of the *COQ3* ORF (corresponding to the proposed mitochondrial targeting sequence) was PCR-amplified from pRS12A-2.5Sma (Clarke et al., 1991) with the primers pCC9 and pML (5′CCCAAGCTTACGCGTTTGTGTTTGAATTGCAAATCTTGA3′), and the product of 122 bp was digested with *Hin*dIII and ligated into the *Hin*dIII site of pAH03 to form the vector pQM. (pAH03 was generated by eliminating the *Mlu*I site in the *CYC1* promoter of pAH01). Primers for amplification of the *Mlu*I/*Mlu*I *ubiG* ORF were as follows: upstream primer pRG1 (5′CCCACGCGTTGTATGAATGCCGAAAAATCGCCGGTA3′); downstream primer pRG2 (5′CCCACGCGTTCACTTATTCTGCGTGTGCAGCAT3′) from pRPB (Wu et al., 1992). The resulting product was digested with *Mlu*I and inserted into the *Mlu*I site of pQM. The recombinant clones containing *ubiG* in the sense orientation were identified by restriction enzyme mapping and named pQMG. High copy number shuttle vectors harboring either *ubiG* (pCHG) or *COQ3* (pCH3) were constructed by digesting pAHG and pAH3 with *Spe*I and *Kpn*I to release the intact *CYC1* promoter−*ubiG* and *CYC1* promoter−*COQ3* DNA fragments, respectively. These DNA fragments were introduced into the *Sma*I site of pCH by blunt-end ligation. pCH was constructed in the same way as pAH01 except that the high copy number vector pRS426 (Christianson et al., 1992) was used instead of pRS316.

Mitochondrial Import Assay. In vitro transcription template plasmids pAH3α and pQMGα were constructed as follows. The *Eco*RV to *Xho*I 1 kb insert containing the yeast *COQ3* ORF from pAH3 was end-filled and inserted via blunt-end ligation into the *Sma*I site of pRS316. A clone which contained this insert downstream of the T7 promoter in the sense orientation was designated as pAH3α. pQMGα contains the mitochondrial leader-ubiG ORF downstream of the T7 promoter and was constructed by excising the 380 bp *CYC1* promoter in pQMG by digestion with *Eco*RI and religation. pAH3α and pQMGα were linearized with *Xho*I and provided templates for the Promega Ribomax Large Scale RNA Production *in vitro* transcription reactions. mRNAs were then translated using Promega Flexi rabbit reticulocyte lysate system in the presence of [^{35}S]methionine from Amersham Life Sciences (1000 Ci/mmol, at final concentration of 0.75 μM). The mRNAs and the synthesized polypeptides were stored at −80 °C. Mitochondria were isolated from W303-1B, and import reactions performed according to Yaffe (1991), except that ATP, phosphoenolpyruvate, and pyruvate kinase concentrations were twice those specified. The import reactions contained 6 μL of radiolabeled *in vitro* translation product and mitochondria (200 μg of protein) and were allowed to proceed for 30 min at 30 °C. At the end of the import, mitochondria were isolated and washed once as described (Yaffe, 1991).

Construction of UbiG Null Mutants. The *Acc*I site in pBlueScript II SK(+) (Stratagene) was removed by *Acc*I digestion, end-repairing, and religation to generate pBSA. A fragment of *E. coli* genomic DNA (3.86 kb) containing the *ubiG* locus and flanking regions was generated by PCR from *E. coli* JC7623 genomic DNA with the primers pG5F (5′CCCGAATTCCCCACTGCCAGCATATTGCAACGC3′, from 4100 to 4087, corresponding to a region 3034 bp upstream of the ubiG ATG codon; Hussain et al., 1987) and pG3F2 (5′CCGGAATTCCCGGATGCGGCGTAAACGCCTTAT3′, from 1674 to 1653, corresponding to a region 107 bp downstream of the ubiG stop codon; Wu et al., 1992). The 3.86 kb PCR product was inserted via blunt-end ligation into the end-filled *Eco*RI site of pBSA to generate pBSG. Disruption of the *ubiG* locus in pBSG was constructed by partial digestion with *Acc*I to generate the linearized plasmid, and the 1.2 kb *Sma*I fragment of the Tn5 kanamycin resistance cassette of pUC4-KIXX (Pharmacia Biotech) was inserted via blunt-end ligation. Clones which contained the cassette within the *ubiG* locus were identified by restriction enzyme digestion, and the desired disruption construct (pBGK) was then linearized with *Bam*HI and used to transform *E. coli* JC7623. Kan^R and Amp^S transformants that failed to grow on SDM were selected and designated as JCΔG after verifying the disruption of the *ubiG* locus by PCR and Southern hybridization. The disrupted *ubiG* locus was transduced into *E. coli* HW272 with P1*vir* phage according to Miller (1992); the transductant obtained, GD1, failed to grow on SDM and was Kan^R. The disrupted *ubiG* locus of GD1 was confirmed by PCR and Southern hybridization.

Lipid Extraction and Analysis of E. coli Q_8 and Q_8 Intermediates. 1 L of GDM broth, containing 0.65 μCi of *p*-[U-^{14}C]hydroxybenzoic acid (365 Ci/mol, prepared as described; Poon et al., 1995), was inoculated with a 5 mL overnight culture of *E. coli* GD1. After incubation at 37 °C with shaking (350 rpm) for 16−20 h, the bacteria were harvested, and lipids were extracted and concentrated to 1 mL/L of original culture as described (Poon et al., 1995). Radiolabeled lipid extracts were separated by chromatography on C18 SepPak columns (Waters), which had been prewashed with 10 void volumes of acetonitrile. An aliquot of *E. coli* lipid extract (100 μL) was mixed with 900 μL of

acetonitrile, loaded onto the column, and eluted stepwise with 10 column volumes of (1) acetonitrile, (2) 85:15 acetonitrile/isopropanol, (3) 7:3 acetonitrile/isopropanol, and (4) isopropanol. Most of the radioactivity (70%) was eluted in the 85:15 acetonitrile/isopropanol fraction. The C18 SepPak column fractions were then further purified by reverse-phase HPLC as described above except the mobile phase consisted of a linear gradient of acetonitrile and isopropanol: samples were injected in 100% acetonitrile, and the percentage of acetonitrile decreased 1% per minute linearly to 50% (flow rate of 1 mL/min). UV absorbance at 272 nm was monitored, and 1 mL fractions were collected. The radioactivity in each fraction was determined, and the radioactive fractions were concentrated under nitrogen, resuspended in heptane and subject to mass spectrometry analysis as described (Poon et al., 1995).

Chemical Synthesis of Q-Intermediates. All reagents for organic synthesis were purchased from Aldrich and used as received unless otherwise stated. Dichloromethane, *N*,*N*-dimethylformamide, hexamethylphosphoric triamide, methanol, and triethylamine were distilled from calcium hydride.

3-Bromocatechol (***11***). 2-Bromophenol was transformed into **11** in two steps using previously described methods for a Reimer−Tiemann *ortho*-formylation (Wynberg et al., 1982) of the phenol, followed by a Dakin oxidation of the corresponding aldehyde (Wreide et al., 1987; Hocking, 1973). ^{1}H NMR ($CDCl_3$, 360 MHz) δ: 5.50 (br s, 2H); 6.74 (t, 1H, J = 8.1 Hz); 6.88 (dd, 1H, J = 8.0, 1.3 Hz); 7.01 (dd, 1H, J = 8.2, 1.4 Hz). ^{13}C NMR ($CDCl_3$, 90 MHz) δ: 109.99; 114.91; 121.95; 123.34; 140.03; 140.47. IR (thin film on NaCl) (cm^{-1}): 3422; 2923; 1701; 1601; 1485; 1462; 1375; 1329; 1254; 1159; 879; 764.

1,2-Diacetoxy-3-bromobenzene (***12***). 3-Bromocatechol **11** (250 mg, 1.3 mmol) was suspended in dichloromethane (2.5 mL). Triethylamine (0.64 mL, 4.6 mmol) and acetic anhydride (0.38 mL, 4.0 mmol) were then added, followed by 4-(dimethylamino)pyridine (ca. 3 mg). The reaction was allowed to proceed overnight at room temperature (16 h). The reaction was then quenched with H_2O (3 mL), and the dichloromethane was removed *in vacuo*. The aqueous phase was extracted with ethyl acetate (2 × 5 mL), and the combined organic layers were washed with brine (5 mL), dried over magnesium sulfate, filtered and concentrated. Silica gel chromatography (R_f 0.4, 8:2 hexanes/ethyl acetate) was performed to obtain 283 mg (79% yield) of white crystalline product **12**. Mp: 63−64 °C. ^{1}H NMR ($CDCl_3$, 360 MHz) δ: 2.25 (s, 3H); 2.32 (s, 3H); 7.14 (m, 2H); 7.46 (dd, 1H, J = 7.4, 2.3 Hz). ^{13}C NMR ($CDCl_3$, 90 MHz) δ: 20.27; 20.55; 117.44; 122.73; 127.21; 130.35; 140.67; 143.63; 167.23; 167.96. MS EI (*m/z*) calcd $C_{10}H_9Br$ (M^+), 271.9684; found, 271.9680. IR (thin film on NaCl) (cm^{-1}): 3080; 2950; 1765; 1452; 1373; 1254; 1209; 1014; 912; 853; 826.

1,2-Diacetoxy-3-farnesylbenzene (***13***). Tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$: 2 mg, 0.002 mmol] was dissolved in hexamethylphosphoric triamide (HMPA: 0.2 mL) in a Schlenk tube under Ar in a glovebox. 1,2-Diacetoxy-3-bromobenzene **12** (40 mg, 0.146 mmol) was then dissolved in HMPA (0.15 mL), and the resulting solution was added to the solution containing $Pd(PPh_3)_4$. A solution of farnesyl tributylstannane (Still, 1978) (109 mg, 0.219 mmol) dissolved in HMPA (0.2 mL) was then added to the reaction mixture. The reaction vessel was sealed under Ar, removed from the glovebox, and heated at 65 °C in a sand bath for 20 h (Stille, 1986). The reaction mixture was quenched with saturated ammonium chloride (2 mL) and extracted with ether (3 × 2 mL). The etherial layers were washed with H_2O (2 mL), brine (2 mL), dried over magnesium sulfate, filtered, and concentrated. Silica gel chromatography was performed (R_f 0.5, 9:1 hexanes/ethyl acetate) to give 40 mg (69% yield) of pale yellow oil **13**. ^{1}H NMR ($CDCl_3$, 360 MHz) δ: 1.60 (s, 6H); 1.68 (s, 6H); 2.04 (m, 8H); 2.28 (s, 3H); 2.29 (s, 3H); 3.26 (d, 2H, J = 7.2 Hz); 5.10 (m, 2H); 5.23 (m, 1H); 7.03 (dd, 1H, J = 7.9, 1.7 Hz); 7.15 (m, 2H). ^{13}C NMR ($CDCl_3$, 90 MHz) δ: 15.98; 16.09; 17.64; 20.28; 20.65; 25.65; 26.42; 26.67; 28.44; 39.59; 39.66; 120.87; 120.90; 123.91; 124.30; 126.20; 127.05; 131.24; 135.15; 135.53; 137.32; 140.51; 142.37; 168.07; 168.28. MS EI (*m/z*) calcd $C_{25}H_{34}O_4$ (M^+), 398.2457; found, 398.2451. IR (thin film on NaCl) (cm^{-1}): 2963; 2922; 2855; 1771; 1468; 1372; 1257; 1169; 1018; 907; 828.

2-Farnesyl-6-hydroxyphenol (***3***). Compound **13** (80 mg, 0.18 mmol) was dissolved in anhydrous methanol (4 mL). Solid potassium carbonate (53 mg, 0.39 mmol) was then added, and the reaction mixture was stirred for 10 min (Plattner et al., 1972). The reaction was quenched by acidification with 10% HCl (to pH 5) and then extracted with ethyl acetate (2 × 5 mL). The combined extracts were washed with H_2O (5 mL) and then with brine (5 mL), dried over magnesium sulfate, and filtered. After concentration, 58 mg (92% yield) of yellow oil **3** was obtained. TLC: (R_f 0.4, 8:2 hexanes/ethyl acetate). ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.61 (s, 6H); 1.69 (s, 3H); 1.79 (s, 3H); 2.08 (m, 8H); 3.38 (d, 2H, J = 7.2 Hz); 5.09 (m, 2H); 5.37 (m, 1H); 5.44 (br s, 2H); 6.72 (m, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 16.09; 16.22; 17.72; 25.71; 26.35; 26.73; 29.79; 39.69; 39.74; 113.29; 120.68; 121.48; 21.75; 123.65; 124.35; 127.48; 131.40; 135.74; 138.82; 142.06; 144.24. MS EI (*m/z*) calcd $C_{21}H_{30}O_2$ (M^+), 314.2246; found, 314.2249. IR (thin film on NaCl) (cm^{-1}): 3428; 2963; 2922; 2853; 1595; 1475; 1451; 1377; 1281; 1186; 910; 733. UV λ_{max} (nm): 274, 232.

2-Farnesyl-6-methoxyphenol (***6***). To a solution of 2-farnesyl-6-hydroxyphenol **3** (58 mg, 0.18 mmol) in *N*,*N*-dimethylformamide (2 mL), imidazole (36 mg, 0.53 mmol) was added, followed by *tert*-butyldimethylsilyl chloride (80 mg, 0.53 mmol). The reaction was allowed to proceed for 20 h at room temperature. The reaction was quenched with H_2O (2 mL) and extracted with ethyl acetate (2 × 2 mL). The combined organic extracts were washed with H_2O (2 × 2 mL), brine (2 mL), dried over magnesium sulfate, filtered, and concentrated. The crude oil was washed through a plug of silica gel (R_f 0.7, 12:1 hexanes/ethyl acetate) to remove polar impurities. After concentration, the silylated intermediate (47 mg), was obtained as a yellow oil.

This silylated material (47 mg, 0.086 mmol) was then dissolved in *N*,*N*-dimethylformamide (0.5 mL). Anhydrous potassium fluoride (10 mg, 0.172 mmol) was added, followed by methyl iodide (6.4 μL, 0.103 mmol) (Sinhababu et al., 1987). The reaction mixture was irradiated with ultrasound under Ar for 2 h at room temperature. The mixture was then poured into H_2O (1 mL) and extracted with ether (2 × 2 mL). The etherial layers were washed with H_2O (1 mL), brine (1 mL), dried over magnesium sulfate, and filtered. After concentration, the crude mixture was separated using silica gel chromatography (R_f 0.5, 12:1 hexanes/ethyl acetate) to give 8 mg (28% yield) of yellow oil **6**. ^{1}H NMR (C_6D_6,

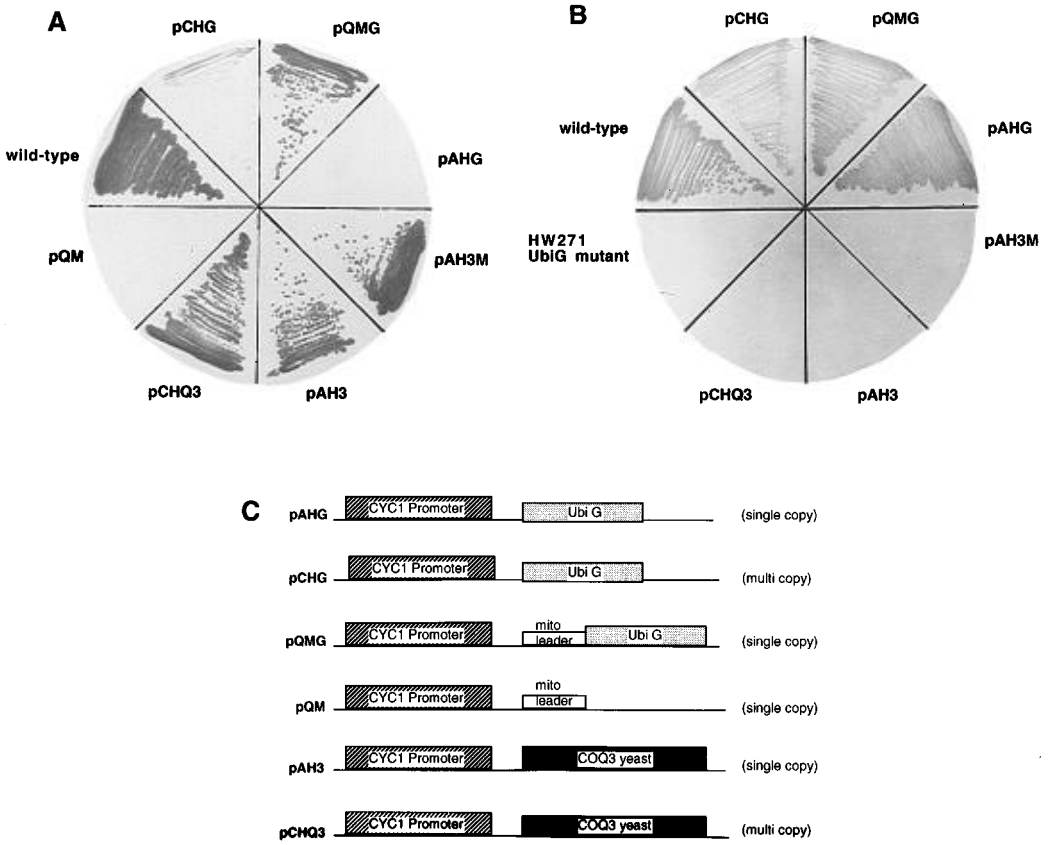

FIGURE 2: Rescue of respiration in *coq3*Δ mutant yeast by the *E. coli ubiG* gene requires the presence of a mitochondrial leader sequence. (A) Wild-type yeast (JM45) or *coq3*Δ yeast (JM45Δcoq3) transformed with the designated plasmids were streaked onto YPG plate agar media containing glycerol as the sole carbon source and incubated at 30 °C for 4 days. (B) Wild-type *E. coli* (HW272), or *ubiG* mutant *E. coli* (HW271) transformed with the designated plasmids were streaked onto SDM plate agar media containing succinate as the sole carbon source and incubated at 37 °C for 1 day. (C) Plasmids were constructed as described in Materials and Methods, and their maintenance in yeast is designated as either single-copy or multicopy.

360 MHz) δ: 1.55 (s, 6H); 1.67 (s, 3H); 1.59 (s, 3H); 2.09 (m, 8H); 3.14 (s, 3H); 3.61 (d, 2H, J = 7.3 Hz); 5.25 (m, 2H); 5.57 (m, 1H); 5.60 (s, 1H); 6.39 (d, 1H, J = 8.0 Hz); 6.76 (t, 1H, J = 7.9 Hz); 6.91 (d, 1H, J = 7.8 Hz). ^{13}C NMR (C_6D_6, 90 MHz) δ: 15.88; 15.96; 17.51; 25.62; 26.73; 26.96; 28.22; 39.92; 39.95; 55.10; 108.33; 119.93; 122.18; 122.91; 124.57; 124.79; 1 C under benzene signal; 130.80; 134.70; 135.73; 143.84; 146.42. Regiochemistry of compound **6** was confirmed by NOESY ^{1}H 2-D NMR coupling of the methoxy protons to the *ortho* aromatic ring protons, as observed in C_6D_6 ($CDCl_3$ did not provide resolution of the aromatic protons). MS EI (*m/z*) calcd $C_{22}H_{32}O_2$ (M^+), 328.2402; found, 328.2398. IR (thin film on NaCl) (cm^{-1}): 3414; 2965; 2924; 2855; 1591; 1471; 1437; 1287; 1190; 1155; 1001; 750. UV λ_{max} (nm): 269, 232.

Compound **14** (2 mg) was also obtained. ^{1}H NMR ($CDCl_3$, 360 MHz) δ: 1.55 (s, 3H); 1.59 (s, 3H); 1.67 (s, 3H); 1.72 (s, 3H); 2.05 (m, 8H); 3.37 (d, 2H, J = 7.1 Hz); 3.79 (s, 3H); 5.10 (m, 2H); 5.28 (m, 1H); 5.56 (s, 1H); 6.71 (d, 1H, J = 7.8 Hz); 6.81 (d, 1H, J = 8.0 Hz); 6.94 (t, 1H, J = 7.8 Hz).

In Vitro Methyltransferase Assay. Each reaction mixture (250 μL) contained 0.05 M sodium phosphate, pH 7.0, 1.0 mM $ZnSO_4$, 1.0 mM substrate (2-hydroxy-5-farnesylphenol or 3,4-dihydroxy-5-farnesylbenzoic acid in methanol), and 100 μL of whole cell extract (protein concentration 1−1.5 mg/mL determined by the BCA method, Pierce Chemical Co.) or permeabilized cells (4−5 mg dry weight cell per mL). *E. coli* cell extracts were prepared as described (Leppik et al., 1976) except that cells were disrupted by French press and the total lysate was used. To prepare permeabilized *E. coli* (Leive, 1965), freshly harvested cells were resuspended in 0.05 M sodium phosphate, pH 7.0, 0.5 mM sodium EDTA, at 2 mL/gram of wet weight cells and incubated at 37 °C for 10 min. The cells were then washed with 0.05 M sodium phosphate, pH 7, once before the addition of methyltransferase assay reaction mix. Reactions were started with the addition of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine to a final concentration of 20 μM (NEN-DuPont, 84.1 Ci/mmol; specific activity was adjusted to 560 mCi/mmol with unlabeled *S*-adenosyl-L-methionine; ϵ 15 200 M^{-1} cm^{-1}, 256 nm, pH 1; Dawson et al., 1986). After incubation (37 °C, 1 h) the reaction was terminated by the addition of 2 μL of acetic acid, and lipids were extracted with chloroform (0.5 mL, twice), concentrated, and resuspended in methanol. The resulting samples were analyzed on an HPLC reverse-phase column (Alltech Lichrosorb C-18, 5 μm, 4.6 × 250 mm) with 9:1 methanol/water as a mobile phase and a flow rate of 1 mL/min, and fractions (1 mL) were analyzed for radioactivity by scintillation counting as described (Poon et al., 1995). The counting efficiency of ^{3}H was 40.5%.

RESULTS

Complementation of Yeast coq3 Mutant by the E. coli ubiG Gene. To test whether the *E. coli ubiG* gene could functionally substitute for the yeast *COQ3* methyltransferase, the yeast expression constructs depicted in Figure 2 were tested for the ability to restore growth of *coq3*Δ mutant yeast

on glycerol (a nonfermentable carbon source). The *E. coli ubiG* gene, when expressed from a yeast promoter, restored respiration (as manifested by growth on glycerol) in the yeast *coq3* mutant provided that the UbiG polypeptide contained an amino terminal mitochondrial import leader sequence (pQMG, Figure 2A). The 35-amino acid mitochondrial leader sequence used to target the UbiG polypeptide was derived from the amino terminal sequence of the yeast Coq3 polypeptide (Clarke et al., 1991). This sequence is characteristic of mitochondrial leader sequences (Hartl et al., 1989) and contains a 3-amino acid motif present in the amino terminus of polypeptides targeted to the mitochondrial matrix (Hendrick et al., 1989). The *E. coli ubiG* gene expressed without the leader sequence from the yeast *CYC1* promoter fails to restore growth on glycerol in the yeast *coq3* mutant when present in single copy (pAHG) and rescues only inefficiently when present in yeast multicopy vectors (pCHG). Overexpression of leaderless polypeptides has been shown to circumvent the requirement for the mitochondrial import sequence (Dircks & Poyton, 1990). The pQMG construct failed to restore growth on glycerol in any of the other yeast *coq* mutants (*coq4*−*coq8*) (data not shown). All of the *ubiG* constructs (pCHG, pQMG, and pAHG) restored respiration (as manifested by growth on succinate) in the *E. coli ubiG* mutant strain HW271 (Figure 2B), indicating the presence of a functional *ubiG* gene and the function of the yeast *CYC1* promoter in *E. coli*. As shown in Figure 2B, the *COQ3* gene from *S. cerevisiae* did not restore growth on succinate in the *E. coli ubiG* mutant. The inability of the yeast *COQ3* gene to rescue the *ubiG* mutants may result either from a more stringent substrate specificity of Coq3p or from inefficient expression in *E. coli*. Based on these observations, the *E. coli ubiG* gene appears to encode a promiscuous *O*-methyltransferase, capable of methylating both **4** in yeast and **9** in *E. coli*. These findings indicate that the *O*-methyltransferase reactions occur in the mitochondrion and suggest that the UbiG polypeptide may catalyze both the first and last *O*-methyltransferase steps in Q biosynthesis in *E. coli.*

Mitochondrial Localization of Coq3p. *In vitro* mitochondrial import assays were performed to determine whether the putative mitochondrial leader sequence of Coq3p functions to direct mitochondrial import. The Coq3p ^{35}S-labeled *in vitro* translation products (Figure 3A, lane 1) generated a 36.8 kDa precursor and two smaller products (29 and 27 kDa); the 29 kDa polypeptide may correspond to initiation of translation at an internal ATG start codon. Upon incubation with mitochondria, a new polypeptide corresponding to the mature form of Coq3p (32.6 kDa) accumulated (lane 2). The mature Coq3p was protected from proteinase K (lane 4), and the addition of Triton X-100 rendered the mature Coq3p susceptible to proteinase K treatment (lane 5). The insensitivity to exogenous protease and the observed proteolytic processing of Coq3p suggested that it was imported into the mitochondria. The accumulation of mature Coq3p was dependent on the mitochondrial membrane potential as it was abolished by the addition of the uncoupler valinomycin (lanes 6 and 7). Similarly, the mitochondrial leader−UbiG fusion protein was translocated into mitochondria in a membrane potential dependent fashion and was resistant to exogenous protease digestion upon the import (Figure 3B). However, cleavage of this fusion protein was not detected.

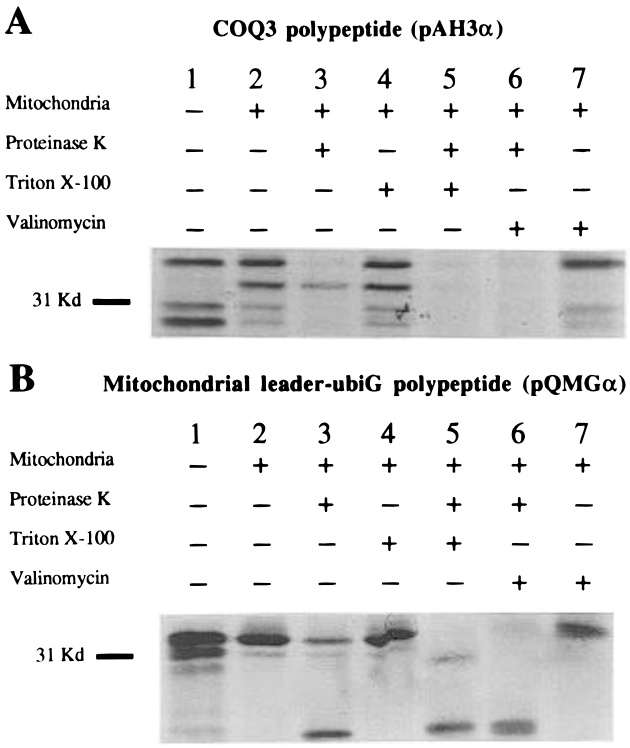

FIGURE 3: Yeast Coq3 leader sequence directs mitochondrial import of Coq3 and UbiG. *In vitro* mitochondrial import of yeast Coq3p (A) and UbiG modified with an amino terminal mitochondrial leader sequence (B). Lane 1, 1 μL *in vitro* translation product; lane 2, *in vitro* translation product incubated with mitochondria; lane 3, same as lane 2 but proteinase K was added to the resuspended mitochondria at final concentration of 50 μg/mL, and after incubation at 0 °C for 20 min phenylmethylsulfonyl fluoride was added (1 mM final concentration); lane 4, same as lane 2 but reisolated mitochondria disrupted with 1% Triton X-100; lane 5, following import the isolated mitochondria were treated with both 1% Triton X-100 and 50 μg of proteinase K/mL; lane 6, mitochondria incubated with *in vitro* translation product in the presence of valinomycin (10 μg/mL final concentration) and the isolated mitochondria were then treated with 50 μg of proteinase K/mL; lane 7, same as lane 6 but no proteinase K treatment. Each sample was mixed with sample buffer, heated at 100 °C for 5 min, and subjected to polyacrylamide gel electrophoresis and autoradiography as described under Materials and Methods.

Construction and Analysis of an E. coli ubiG Null Mutant. On the basis of the rescue of *coq3*Δ yeast by *ubiG*, it seemed likely that UbiG may catalyze both *O*-methyltransferase steps in *E. coli*. One prediction of this hypothesis is that *ubiG* null mutants would not produce demethyl-Q (**9**) and instead would be expected to accumulate compound **3**. In order to test this hypothesis, we generated a *ubiG* disruption mutant in *E. coli* (strain GD1). We have shown that GD1 is respiration defective; it does not grow on a defined medium with succinate as the sole carbon source, an expected result of Q-deficiency. Transformation of GD1 with the *E. coli ubiG* gene on a plasmid restores respiration (growth on succinate) and the production of Q_8 (data not shown). Q and Q intermediates were radiolabeled by supplementing the growth media with *p*-[U-^{14}C]-hydroxybenzoic acid, which is thought to be a committed precursor in the pathway. Analysis of *p*-[U-^{14}C]hydroxybenzoate radiolabeled lipid extracts from the GD1 mutant showed that this mutant fails to produce Q or any quinone-containing intermediate (Figure 4). The *ubiG* disruption strain GD1 did not yield recoverable **3**; instead **2** was present at ∼20% of the wild-type Q_8 level (fractions 35−37; M^+, $C_{46}H_{70}O$ requires theoretical mass of

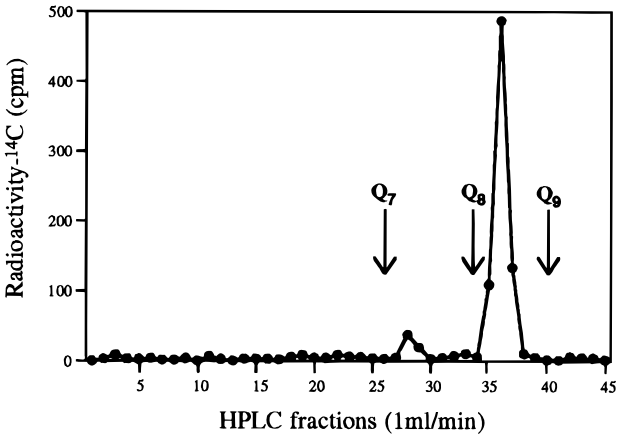

FIGURE 4: Reverse-phase HPLC of *p*-[U-^{14}C]hydroxybenzoic acid-labeled *E. coli* GD1 lipid extracts. An aliquot of lipid extract from the *ubiG* disruption mutant GD1 (corresponding to 3×10^9 cells) was injected onto the HPLC and is presented as ^{14}C radioactivity in each 1 min fraction as determined by scintillation counting. The only Q intermediate identified by mass spectrometry was **2** ($n = 8$) present in fractions 35−37. The arrows indicate the retention times of standards Q_7, Q_8, and Q_9.

638.542667; observed mass 638.543120, ppm −0.7; tropylium ion, C_7H_7O, was the observed base peak at 107 *m/z*). Analysis of fraction 28 did not yield meaningful mass spectrometry data. Attempts to derivatize (and hence stabilize) intermediate **3** by treatment of the crude lipid extract with diazoethane generated only ethylated-**2** (M^+, $C_{48}H_{74}O$, theoretical mass 666.573968; observed mass 666.573943, ppm −3.0). Similar modification of a synthetic farnesylated analog of intermediate **3** (Figure 5) with either diazoethane (or diazomethane) generated the expected diethylated (or dimethylated) product (data not shown). These data indicate that GD1 is not a leaky mutant (unlike the *ubiG* mutant AN151, which was reported to accumulate Q_8 and demethyl-Q_8 as well as 2-octaprenylphenol; Stroobant et al., 1972). Another *E. coli ubiG* mutant HW271 (a *ubiG* point mutant; Wu et al., 1992) was analyzed; (the Q intermediates accumulating in lipid extracts of HW271 have not been previously reported). Interestingly, in extracts prepared from this mutant we detect only **2** (data not shown), as we found for the disruption mutant GD1. It seems likely that the AN151 mutant (*ubiG423*) contains a particular example of *ubiG* mutant allele, allowing partial activity and thus production of demethyl-Q_8 and Q_8. These results are consistent with the idea that the UbiG methyltransferase may catalyze both *O*-methyltransferase steps. However, if this is the case, it is not clear why we fail to detect compound **3**. It is important to note that compound **3**, a catechol intermediate, may be very unstable (Waite, 1990).

O-Methylation Assays. To determine whether the *E. coli* containing the UbiG polypeptide recognizes compounds **3** and **4** as substrates for *O*-methylation (in addition to compound **9**), farnesyl analogs of these Q biosynthetic intermediates were synthesized chemically [Shepherd et al. (1996) and Figure 5]. These compounds were tested as substrates in *in vitro* methylation assays (Figure 6). *E. coli* wild-type (HW272) whole cell extracts contained high activity, methylating both the farnesylated analog of **3** (panel A, 279 pmol of product/mg of protein/h) and **4** (panel B, 213 pmol of product/mg of protein/h). The radioactive methylated products co-eluted with the corresponding chemically synthesized methylated products (**5** and **6**). Leppik et al. (1976) performed a similar study using demethyl-Q_8 (**9**) as substrate and determined the methyltransferase activity of wild-type (AN3311) extract to be 680 pmol Q_8 formed/mg of protein/hr. *In vitro* assays performed with whole cell extracts of AN151 (*ubiG423*) or GD1 (ΔubiG) yielded no detectable methyltransferase activity over background. On the basis of the analysis of intermediates generated *in vivo* (Stroobant et al., 1972), AN151 (*ubiG423*) might be expected to have higher activity than GD1 (ΔubiG). This was in fact observed when permeabilized cells were employed in the methylation assays instead of cell extracts. Following permeabilization with EDTA, intact cells were tested in the methyltransferase assay with the farnesyl derivative of **3** (Figure 7). HW272 again showed high activity, 165 pmol of product/mg of protein/h or 44 pmol of product/mg of dry weight cells/h. GD1 (ΔubiG) under this condition showed no activity, while AN151 (*ubiG423*) had appreciable activity, about 1.54% of the wild-type level (see inset). No radiolabeled product corresponding to the unnatural isomer, 2-methoxy-3-farnesylphenol (iso-**6**; compound **14** in Figure 5) was detected. Since Q_3 and **6** have similar retention times on the HPLC system in Figure 6, samples were treated with sodium borohydride to generate Q_3H_2 (the hydroquinone form of Q_3) which elutes earlier. Such treatment of the extracted lipids of HW272 produced no change in the retention time of the radiolabeled products and indicated that Q_3 was not produced in this system (data not shown). These *in vitro* assays show that the presence of an intact *ubiG* gene is required for the first *O*-methylation in the *E. coli* Q biosynthetic pathway. In addition, this activity is present but only partially active in AN151, the leaky *E. coli ubiG* mutant.

DISCUSSION

The data presented here show that the UbiG methyltransferase is required to carry out both *O*-methylation steps in *E. coli*. The *in vitro* methyltransferase assays, both with whole cell extracts and with permeabilized cells, provide evidence that UbiG catalyzes the step converting compound **3** to compound **6** in *E. coli*. UbiG may also act as a promiscuous methyltransferase in converting the yeast intermediate (compound **4**) to **5** as demonstrated by *in vitro* assays, and also by the ability of *ubiG* to restore growth of the yeast *coq3* mutant on a nonfermentable carbon source. These studies suggest that the length of the isoprene tail does not play a crucial role in substrate recognition since $n = 6$ in the Q intermediates of *S. cerevisiae* and $n = 3$ in the chemically synthesized substrate analogs. Thus it seems likely that UbiG is involved in the methylation of three distinct substrates. Leppik et al. (1976) showed that UbiG was required for the *O*-methylation converting demethyl-Q (**9**, $n = 8$) to Q. In our assays, UbiG did, however, show specificity as it did not produce detectable amounts of the unnatural isomer 2-methoxy-3-farnesylphenol (compound **14** or iso-**6**). Methyltransferase assays with EDTA-permeabilized cells indicated the presence of a low but significant level of methyltransferase activity in the *ubiG* mutant AN151 (*ubiG423*), while no activity was detected in the *ubiG* disruption strain GD1. This result is consistent with the previous analysis of the AN151 *ubiG* mutant as being leaky, since in addition to detection of compounds **2** and **9**, Q_8 was also detected in extracts of AN151 at about 5% the level of wild-type cells (Stroobant et al., 1972).

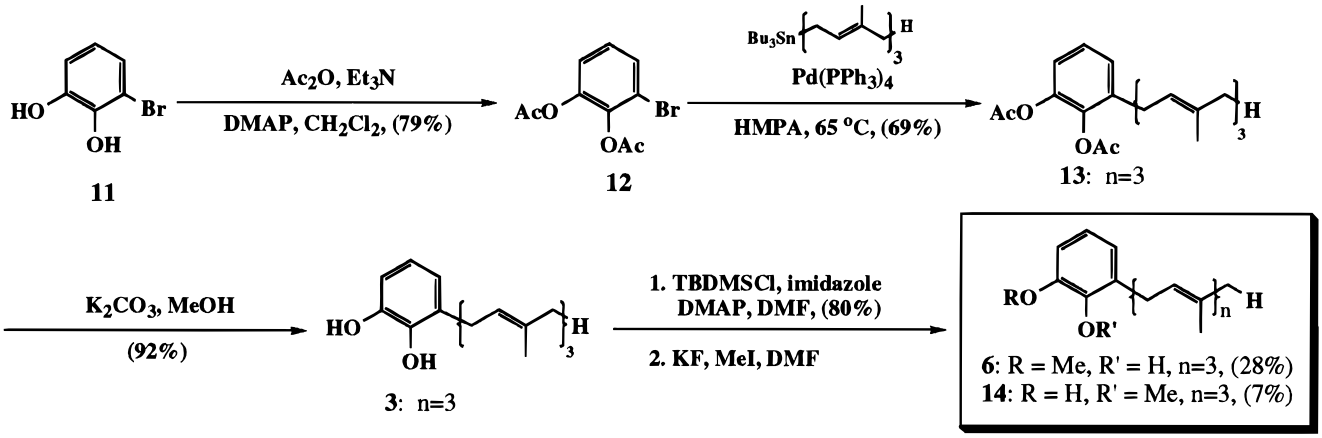

FIGURE 5: Chemical synthesis of farnesylated analogs of Q intermediates. Experimental details are presented under Materials and Methods.

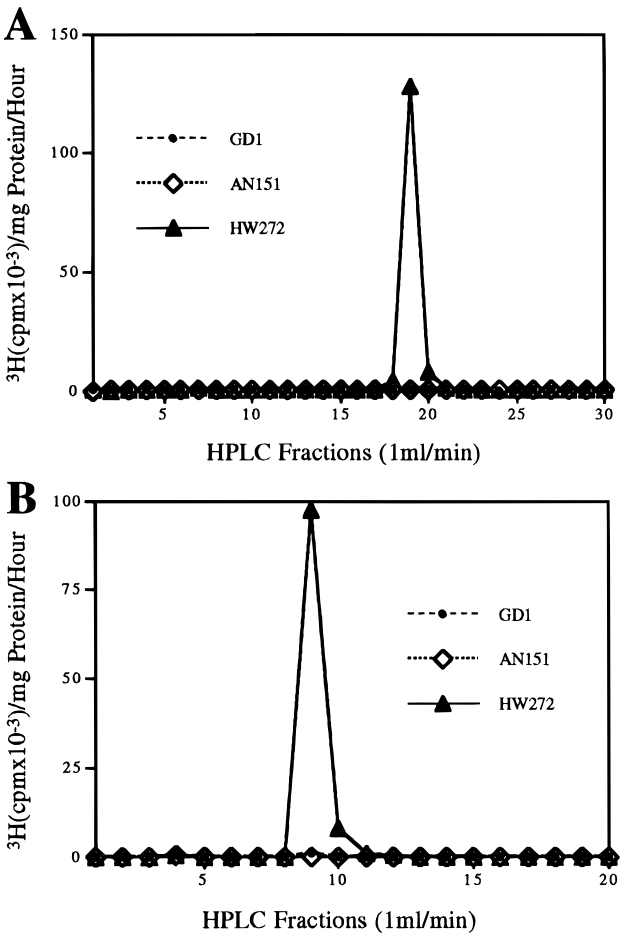

FIGURE 6: Synthetic analogs of yeast and *E. coli* Q intermediates serve as methyl-acceptor substrates for *E. coli* UbiG. *E. coli* cell extracts from HW272 (wild-type), AN151 (*ubiG* leaky mutant), or GD1 (*ubiG* disruption mutant) were prepared, and *in vitro* methyltransferase assays were performed with addition of either **3** (n = 3; panel A) or **4** (n = 3; panel B) as methyl-acceptor substrates and *S*-adenosyl-[*methyl*-^{3}H]-L-methionine. Radioactivity associated with the lipid extracts of the *in vitro* assays was separated by HPLC. The standards corresponding to the methylated products, 2-methoxy-6-farnesylphenol (**6**) and 3-methoxy-4-hydroxy-5-farnesylbenzoic acid (**5**), eluted at fractions 19 and 9, respectively. No radiolabel was detected in fraction 9 in the absence of substrate; the background radioactivities contained in fraction 19 of AN151 and GD-1 in the absence of substrate were 102 and 189 cpm/mg of protein/h, respectively.

Curiously, compound **3** was not detected in the AN151 *ubiG* mutant (Stroobant et al., 1972) and no *E. coli* mutant has been reported which accumulates compound **3**. In fact

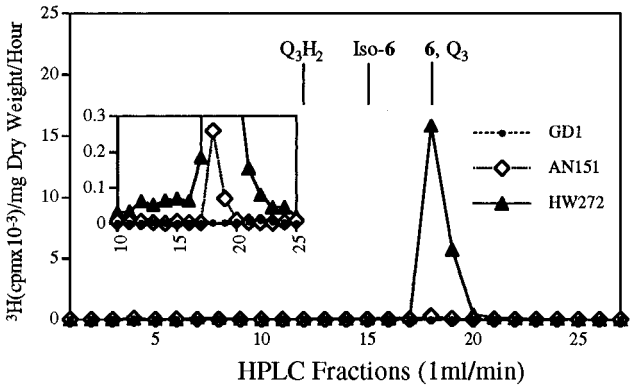

FIGURE 7: *In vitro* methylation assays with permeabilized *E. coli* cells reveal an impaired *O*-methyltransferase activity present in the leaky *ubiG* mutant AN151. HPLC analysis of *in vitro* methyltransferase assays with permeabilized *E. coli* cells HW272 (wild-type), AN151 (*ubiG* leaky mutant), or GD1 (*ubiG* disruption mutant) and **3** (n = 3) as substrate. Retention times were shifted (approximately 1 min relative to Figure 6) due to the preparation of mobile phase. The elution positions of standards are indicated. Iso-**6** eluted at fraction 15 and corresponds to the unnatural isomer 2-methoxy-3-farnesylphenol (compound **14** in Figure 6). Q_3 eluted at fraction 18 similar to **6**; however, upon reduction by $NaBH_4$ (added directly to the sample in methanol and incubated for 30 min) the retention time of the resulting Q_3H_2 shifted to fraction 13.

this intermediate has not yet been detected, although it is the predicted intermediate located between the steps blocked in *E. coli* mutants *ubiB* and *ubiH* (Gibson,1973). Its absence is notable because Young et al. (1973) examined lipid extracts prepared from both *ubiH* mutants and *met* mutants (methionine limitation experiments) and despite the formation of **2** as the predominant intermediate, **3** was not detected. It was concluded that **3** may not exist as a free intermediate (Gibson, 1973). Our attempts to recover **3** from *E. coli* were also unsuccessful. The only intermediate detected in the *ubiG* disruption strain was compound **2** [this is also the predominant intermediate in the AN151 (*ubiG423*) strain]. However, we consistently observed the loss of about 30% of radiolabel in lipid extracts of *ubiG* mutants, but not wild-type extracts, after SepPak column purification. Elution of the column with acidified solvent (0.1% trifluoroacetic acid) resulted in elution of the radiolabeled material, which appeared to contain only decomposed products by HPLC analysis. The difficulty in isolating **3** may be due to the reactivity of the catechol moiety. The chemistry of catechol-like substances is best appreciated by the fact that these compounds participate in the formation of many of biological

adhesives and varnishes (Waite, 1990). In fact it is possible that the first hydroxylation step in both *E. coli* and *S. cerevisiae* is rate-limiting and that such regulation of the Q biosynthetic pathway results in low levels of **3** in *E. coli* and **4** in eukaryotes. Intermediate **2** is the predominant intermediate in wild-type *E. coli* grown anaerobically and is also present to a lesser extent in wild-type cells under aerobic conditions (Alexander & Young, 1978; Gibson & Young, 1978). In rat and yeast, compound **1** is the predominant intermediate (Yamamoto et al., 1989, 1990; Kang et al., 1991; Poon et al., 1995). The accumulation of intermediate **2** in *E. coli* and **1** in eukaryotic cells has been interpreted as indicating that the first hydroxylase is rate-limiting or may indicate a high K_m of this hydroxylase for one of its substrates (Poon et al. 1995). It is important to note that different modes of regulation may result from different growth conditions. For example, Sippel et al., (1983) found that compound **4** accumulates in *S. cerevisiae* when grown under conditions of high glucose, which functions to repress Q synthesis and electron transport.

It is perhaps surprising that in the permeabilized *E. coli* cell assay used in this study, none of the radiolabel was converted to Q. Yet it is clear that, despite several studies, an efficient *in vitro* system for the synthesis of Q has not yet been attained. Such *in vitro* assays performed with either *p*-[U-^{14}C]hydroxybenzoate or with 3-polyprenyl[U-^{14}C]-hydroxybenzoate as substrates in combination with isolated mitochondria (or microsomal fractions) from rat liver generate radiolabeled Q very inefficiently (Kalen et al., 1990; Trumpower et al., 1974). Analogous findings have been made with isolated yeast mitochondria (Casey & Threlfall, 1978a). Thus it seems likely that the Q *in vitro* synthetic systems described to date may be inhibited and/or lack key components which greatly limit the metabolism of Q intermediates.

This study also indicates that yeast Coq3p is located mainly in the mitochondria. The yeast Coq3p precursor was imported into mitochondria *in vitro*, and its processing required a membrane potential (Figure 3). Western blotting with polyclonal antibodies to Coq3p shows the size of Coq3p to be that of the mature (processed) form, and subcellular fractionation studies indicate the presence of Coq3p in mitochondria (data not shown). Other organelles may also co-purify with the mitochondria-enriched fraction, hence the presence of Coq3p in other organelles cannot be ruled out. However, it is important to note that rescue of the *coq3* yeast mutant by *ubiG* expressed from a single-copy plasmid required the presence of the mitochondrial leader sequence (Figure 2). Thus the mitochondrial targeting of the *O*-methyltransferase activity is an essential feature of the ability to restore respiration, and hence Q biosynthesis *in vivo*. It seems likely that the restoration of respiration in the *coq3* mutant by leaderless-UbiG on a multicopy plasmid (pCHG) may be due to the entry of small amounts of UbiG into mitochondria. Such multicopy suppression was observed when leaderless-cytochrome *c* oxidase subunit Va present on a multicopy plasmid was found to circumvent the requirement for the mitochondrial leader sequence (Dirks & Poyton, 1990).

Many questions remain regarding the intracellular site(s) of Q biosynthesis in eukaryotes. Studies in yeast on the formation of 3-hexaprenyl-4-hydroxybenzoate (**1**) indicate that both the hexaprenyldiphosphate synthase and the *p*-hydroxybenzoate:polyprenyltransferase activity are associated with mitochondrial fractions (Casey & Threlfall, 1978b). These activities are encoded by the *COQ1* and *COQ2* yeast genes, respectively, and both genes encode polypeptides which contain typical mitochondrial leader sequences (Ashby & Edwards, 1990; Ashby et al., 1992). In addition, the leader sequence of *COQ2* contains the 3-amino acid consensus found in precursors that are processed by two consecutive proteolytic cleavages (Hendrick et al., 1989). However, the mitochondrial localization of these polypeptides has not been demonstrated directly. Recent studies in rat liver suggest that Q biosynthesis may also be localized to the endoplasmic reticulum−Golgi system (Kalen et al., 1987, 1990; Teclebrhan et al., 1995). In these studies 3−4-fold higher levels of *p*-hydroxybenzoate:polyprenyltransferase activity were associated with the endoplasmic reticulum−Golgi system, compared with mitochondria in subcellular fractionations of rat liver. These investigators also found rates of Q synthesis from **1** to be 10-fold higher in microsomes than in mitochondria (Kalen et al., 1990). Conversely, earlier studies by other investigators with rat liver indicate that synthesis of both compound **1** and Q occurs in mitochondria (Momose & Rudney, 1972; Trumpower et al., 1974; Houser & Olson, 1977). The predicted amino terminal sequence of the rat Coq3 polypeptide is typical of mitochondrial leaders and, moreover, was isolated on the basis of its ability to restore Q synthesis in the yeast *coq3* yeast mutant (Marbois et al., 1994). Thus it seems likely that the rat Coq3 polypeptide (like yeast Coq3p) is imported into yeast mitochondria and probably rat mitochondria as well. It is interesting that the *O*-methyltransferase responsible for converting **9** to Q in rat liver was localized to the inner membrane of rat mitochondria (Houser & Olson, 1977). The activity of the rat enzyme required the hydroquinone form of **9** (or the presence of NADH which was used to reduce the oxidized form of **9**). The *E. coli* UbiG methyltransferase also required a reducing agent for activity (Leppik et al., 1976). It is tempting to speculate that rat (and yeast) Coq3p, like UbiG, may catalyze both *O*-methyltransferase steps of Q biosynthesis.

ACKNOWLEDGMENT

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