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Pathway for Ubiquinone Biosynthesis in *Escherichia coli* K-12: Gene-Enzyme Relationships and Intermediates

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Seven ubiquinone-deficient mutants of *Escherichia coli*, each of which accumulates two phenolic precursors of ubiquinone, have been characterized, and the accumulated compounds have been identified. The mutants accumulate small quantities of 2-octaprenyl-6-methoxyphenol, which was isolated and characterized by nuclear magnetic resonance and mass spectrometry, and relatively large amounts of 2-octaprenylphenol, a compound previously identified from *E. coli*. They also accumulate small quantities of a compound identified as 2-(hydroxyoctaprenyl)phenol although the relevance of this compound to the biosynthesis of ubiquinone is not clear. The results of genetic analysis suggest that each of the mutants carries a mutation in a gene (designated *ubiH*) which is located at about min 56 on the *E. coli* chromosome and is co-transducible with the *serA* and *lysB* genes. Based on information obtained from this and previous studies with ubiquinone-deficient mutants, a pathway is proposed for ubiquinone biosynthesis in *E. coli*, and a summary of the known gene-enzyme relationships is given.

Ubiquinones are widely distributed in nature and form a family of 2-polyprenyl-3-methyl-5,6-dimethoxy-1,4-benzoquinones whose members differ in the lengths of their polyprenyl side chains (7). The long hydrocarbon side chains give the ubiquinones a strongly lipophilic character appropriate to their location within membranes, whereas the quinone ring can undergo redox reactions which are important for the role of ubiquinones as carriers in electron transport systems (8). In *Escherichia coli* the predominant homologue of ubiquinone has a side chain consisting of eight isoprene units. To assist in a study of the way in which the synthesis of this membrane component is achieved by the cell, we have isolated a number of mutants of *E. coli* forming little or no ubiquinone. Six classes of ubiquinone mutants and the mapping of the genes concerned on the *E. coli* chromosome have been described previously (6, 23, 30, 31). The *ubiB*, *ubiD*, and *ubiE* genes are closely linked at min 75, whereas the *ubiA*, *ubiF*, and *ubiG* genes are located at min 79, 16, and 42, respectively.

A detailed examination of representatives of the various classes of mutants has allowed the isolation and characterization of the ubiquinone precursors 3-octaprenyl-4-hydroxybenzoic acid, 2-octaprenylphenol, 2-octaprenyl-6-methoxy-1,4-benzoquinone, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, and 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone (6, 23, 31). The structures of the above compounds are shown in Fig. 6. These precursors together with 4-hydroxybenzoate can be arranged to form a logical sequence giving a pathway for ubiquinone biosynthesis from chorismate (23). With the exception of the conversion of 2-octaprenylphenol to 2-octaprenyl-6-methoxy-1,4-benzoquinone, the steps in the sequence are consistent with known biochemical transformation, and the first three reactions have been demonstrated in cell extracts of *E. coli* (6, 14, 30; J. Lawrence, unpublished results).

The present paper describes the properties of the members of a further class of ubiquinone-deficient mutants that are blocked in the conversion of 2-octaprenylphenol to 2-octaprenyl-6-methoxy-1,4-benzoquinone and examines the question of whether 2-octaprenyl-6-hydroxyphenol and 2-octaprenyl-6-methoxy-

phenol are intermediates in this conversion. A pathway is proposed for ubiquinone biosynthesis in *E. coli*.

MATERIALS AND METHODS

Chemicals. Acetone, diethyl ether, and ethyl acetate were redistilled before use. Other chemicals were generally of the highest purity obtainable commercially and were not further purified with the exception of 2-allyl-6-methoxyphenol and 2-methylphenol which were purified by chromatography.

Thin-layer chromatography. Silica Gel plates were used with the following solvents: A, chloroform-light petroleum (70:30, vol/vol); B, ethyl acetate-hexane (25:75, vol/vol). Initial chromatography of lipid extracts was carried out on plates (0.5-mm layer thickness) prepared by using Merck Silica Gel G. Merck precoated (0.25 mm) Silica Gel F254 thin-layer plates were used for subsequent chromatography. Phenolic compounds were located by using diazotized *p*-nitraniline spray (3).

Bacterial strains. All of the strains used were derived form *E. coli* K-12 and are described in Table 1. The ubiquinone-deficient strains were maintained on nutrient agar supplemented with 0.5% glucose.

Isolation of ubiquinone-deficient mutants. Logarithmic-phase cells of strain AB3311 were suspended in sodium citrate buffer (0.1 M, pH 6) and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) for 20 min at 37 C (2). After thorough washing with basal medium (5), they were grown for two generations in complete medium containing glucose (30 mM) to allow phenotypic expression to occur. The resulting cells were then screened by delayed enrichment, and mutants able to grow on glucose but unable to grow on succinate as sole carbon source were isolated. A 1-liter culture of each mutant was grown, and the cells were extracted by using the Soxhlet procedure (see below). Lipid extracts were chromatographed with solvent A, and the plates were examined for the presence of the yellow ubiquinone band (R_f 0.4). About 7% of mutants unable to grow on succinate were ubiquinone deficient.

Media and growth of cells. The glucose-mineral salts medium used and the concentration of supplements have been described previously (23). Cells were grown at 37 C either in 1-liter volumes in shaken flasks or in larger-scale fermentors with aeration and stirring and were harvested in late logarithmic phase.

Preparation of lipid extracts. Two methods were used to prepare lipid extracts, both of which gave efficient extraction of ubiquinone and its lipid precursors. The Soxhlet procedure involved acetone extraction of cells in a Soxhlet extraction apparatus (Quickfit extractor, 60-ml siphoning volume). Up to 30 g (wet weight) of cells was used per extraction thimble, and these were continuously extracted with acetone (150 ml) for 4 h (2 h if less than 6 g cells). The acetone extract was evaporated to dryness on a steam bath, the residue was extracted with 50 ml of light petroleum (60–80 C), and the extract was concentrated.

The second method which involves of Folch extraction (11) is referred to as the cold-extraction procedure. Cells (10 g wet weight) were thoroughly suspended in methanol (70 ml), and the slurry was heated at 55 C for 5 min. Chloroform (140 ml) was added, and the suspension was stirred at 20 C for 20 min and then gravity-filtered through Whatman no. 1 filter paper. One-fifth volume of NaCl solution (0.58%, wt/vol) was added to the filtrate, and then the two were mixed gently and allowed to separate into two phases. The lower phase was evaporated to dryness at 25 C under reduced pressure, and the residue was extracted twice with 10 ml of acetone. This extract was taken to dryness, the residue was extracted twice with 5 ml of light petroleum (60–80 C), and the light petroleum extract was concentrated. For larger quantities of cells, the volumes of solvents were increased proportionally.

Determination of ubiquinone and the octaprenyl phenols. Extracts of lipids from a known weight of cells (about 50 g wet weight) were chromatographed using solvent A. The yellow ubiquinone band was eluted with ethanol, and its concentration was determined spectrophotometrically by reduction with sodium borohyride (9). The octaprenylphenols were eluted with diethyl ether after location of the bands by spraying a narrow sideband with diazotized *p*-nitraniline, and their concentrations were determined from the absorbance at 273 nm. The molar extinction coefficient in diethyl ether of 2-octaprenylphenol (6) was determined and found to be 2,500.

TABLE 1. *Strains of E. coli K-12 used*[a]

Strain	Sex	Relevant genetic loci[b]	Source or other information
AB2154	Hfr	*leu-6, thr-1, metE47, str-8*	
AB3311	Hfr	*metB*$^-$	
AB347	Hfr	*leu*$^-$, *thr*$^-$, *aroC*$^-$	
KL16	Hfr		K. B. Low
AT713	F$^-$	*lysB10, argA21, cysC39, strA9*	A. L. Taylor
AT2475	Hfr	*serA6*	A. L. Taylor
AN83	Hfr	*metB*$^-$, *ubiH424*	Isolated from AB3311 after mutagenesis with NTG[c]
AN160	Hfr	*metB*$^-$, *ubiH429*	Isolated from AB3311 after mutagenesis with NTG
AN164	Hfr	*metB*$^-$, *aroB*$^-$, *str*	
AN172	Hfr	*ubiH424*	Transductant obtained using AN83 as donor and AT2475 as recipient

[a] The other *ubiH* mutants are described in the text.

[b] Genetic nomenclature is that used by Taylor (24).

[c] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

This extinction coefficient was also used for 2-(hydroxyoctaprenyl)phenol and 2-octaprenyl-6-methoxyphenol on the basis of the values found for model compounds in diethyl ether (2-methylphenol, 2,300; 2-allyl-6-methoxyphenol, 2,400). It was found necessary to rechromatograph 2-(hydroxyoctaprenyl)phenol prior to estimation, using solvent B, in order to remove interfering substances. The determination of 2-octaprenyl-6-methoxyphenol was complicated by the presence of menaquinone and desmethylmenaquinone in the sample, as the naphthoquinone band partly overlapped that of the phenol. The contribution of the naphthoquinones was eliminated by adjusting the optical density at 325 nm to zero, using a blank containing material from the phenol-free portion of the naphthoquinone band, and measuring a difference spectrum.

Color test for ortho-substituted octaprenylz phenols. A color test based on the Gibbs reaction for phenols (13) was developed to distinguish 2-octaprenylphenol from 3-octaprenylphenol. An ethanolic solution of 2,6-dichloroquinonechloroimide (20 μliters, 5% wt/vol) was added to the compound to be tested (40 nmol in 0.4 ml of ethanol) and a solution of KOH (20 μliters, 1 M) was added. A positive test, which is the immediate development of a green color, was given by 2-methylphenol and 2-octaprenylphenol, whereas 3-methylphenol and 4-methylphenol gave the same brown color as the reagent blank.

Isolation of 2-octaprenylphenol and 2-(hydroxyoctaprenyl)phenol. Cells (50 g wet weight) of strain AN160 were extracted by using the cold extraction procedure. The lipid extract was chromatographed by using solvent A, and the two phenols were detected by their red-brown color with diazotized *p*-nitraniline. 2-Octaprenylphenol (R_f 0.6) was purified by chromatography as described previously (6). 2-(Hydroxyoctaprenyl)phenol (R_f 0.2) was rechromatographed using solvent B.

Isolation of 2-octaprenyl-6-methoxyphenol. Cells (2.4 kg wet weight) of AN160 were extracted by using the Soxhlet procedure described above, and the resulting lipid extract was chromatographed by using solvent A. The 2-octaprenyl-6-methoxyphenol (R_f 0.8) was only partly separated from the menaquinone-desmethylmenaquinone band in this solvent and was detected by its orange-brown color with diazotized *p*-nitraniline. The compound was eluted with diethyl ether, the eluate was taken to dryness under reduced pressure, and the residue was dissolved in a small volume of absolute ethanol. The naphthoquinones present were then reduced by adding small quantities of a saturated solution of sodium borohydride in absolute ethanol until the absorbance of the solution at 245 nm reached a maximum. The resulting deep violet solution was then chromatographed immediately using solvent A. This procedure was repeated until the 2-octaprenyl-6-methoxyphenol was free from naphthoquinone. The purification of the compound was then completed by further chromatography using solvent B and then solvent A. The compound was eluted with diethyl ether, and the eluate was taken to dryness under reduced pressure and dried at −20 C in a vacuum desiccator over P_2O_5 and paraffin wax. The purified compound (5 mg) was a pale yellow oil at room temperature.

Metabolism of 4-hydroxybenzoate by nongrowing cells in the presence and absence of methionine. A 20-liter aerated culture of strain AN164 (*metB*⁻, *aroB*⁻) was grown at 37 C to mid-logarithmic phase in a glucose-mineral salts medium supplemented with L-phenylalanine (0.2 mM), L-tyrosine (0.2 mM), 4-aminobenzoate (1 μM), thiamine-hydrochloride (1 μM), 2,3-dihydroxybenzoate (0.1 mM), L-tryptophan (0.08 mM), and L-methionine (0.05 mM), the amounts of tryptophan and methionine being chosen so as to limit the growth of the culture. The cells were cooled rapidly, harvested aseptically, washed once with cold, sterile minimal medium, and resuspended to their original volume in glucose-minimal medium containing 4-hydroxybenzoate (0.1 mM). The cell suspension was divided into two lots, methionine (0.1 mM) was added to one only, and both were incubated at 37 C for 6 h with aeration. During this period, there was no increase in the turbidity of either suspension. After incubation, the cells were harvested, and extracts of their lipids were prepared by the cold extraction procedure described above. These extracts were chromatographed using solvent A, and the plates were examined for the presence of ubiquinone and ubiquinone precursors before and after spraying with diazotized *p*-nitraniline.

Genetic mapping. The technique used for interrupted mating experiments was based on that described by Taylor and Thoman (25), and transduction experiments using the generalized transducing phage P1kc were carried out as described by Pittard (22).

Spectroscopy. Mass spectra were obtained with an AEI MS9 double-focus mass spectrometer, nuclear magnetic resonance spectra were obtained with a Varian HA100 spectrometer using tetramethylsilane as internal standard, and ultraviolet spectra were measured using a Cary 15 spectrophotometer.

RESULTS

Seven members of a new class of ubiquinone-deficient mutants have now been characterized. These mutants are differentiated from those previously described (6, 23, 30, 31) on the basis of the compounds they accumulate and by genetic analysis. One of these mutants (AN160) has been used to produce sufficient amounts of the three compounds accumulated by the new class to allow their identification.

Compounds accumulated by the ubiquinone-deficient strain, AN160. Cells of strain AN160 (10 g wet weight) were extracted by the Soxhlet procedure, and the lipid extract was chromatographed with solvent A. No ubiquinone was visible. After spraying of the side band of the chromatogram with diazotized *p*-nitraniline, three prominent colored bands (designated A, B, and C) were observed at R_fs

0.2, 0.6, and 0.8, respectively. Strain AN160 was grown on a larger scale, and the compounds were purified as described in Materials and Methods. Compounds A, B, and C were identified as 2-(hydroxyoctaprenyl)-phenol, 2-octaprenylphenol, and 2-octaprenyl-6-methoxyphenol, respectively (see below).

A comparison was made of the levels of the various compounds in strain AN160, its parent strain AB3311, and strain AN59 (*ubiB*$^-$) (Table 2). Strain AN59 previously has been shown to accumulate 2-octaprenylphenol (6). Of the three strains, only AN160 accumulates 2-(hydroxyoctaprenyl)phenol and 2-octaprenyl-6-methoxyphenol, and these compounds are present at low levels. Both strains AN59 (*ubiB*$^-$) and AN160 accumulate relatively high levels of 2-octaprenylphenol which are equivalent to, or slightly higher than, the amount of ubiquinone formed by AB3311.

Identification of compound A as 2-(hydroxyoctaprenyl)phenol. The ultraviolet spectrum of compound A in diethyl ether and the color given with *p*-nitraniline spray were indistinguishable from those of 2-octaprenylphenol (6). Chromatography with solvent A readily separated compound A (R_f 0.2) from 2-octaprenylphenol (R_f 0.6). These properties suggested that compound A was closely related to 2-octaprenylphenol, but that it probably possessed an additional hydrophilic substituent.

The mass spectrum of compound A (Fig. 1) showed a molecular ion at *m/e* 654, and accurate mass measurement of this peak gave a composition of $C_{46}H_{70}O_2$ (mass found was 654.5360; $C_{46}H_{70}O_2$ requires 654.5375). This composition corresponds to that of an octaprenylphenol which contains an additional oxygen substituent. In the spectrum of compound A, no major peaks below *m/e* 654 were found with a composition corresponding to fragments containing two oxygen atoms. The fragmentation pattern observed is similar to that of 2-octaprenylphenol (6), except for the additonal presence of peaks at *m/e* 636 and *m/e* 547. The M-18 peak at *m/e* 636 strongly suggests that the additional oxygen function present in compound A is an hydroxyl group since many classes of alcohols dehydrate readily in the mass spectrometer (4). Although a number of mono- and dihydroxybenzene derivatives yield M-18 peaks (1), these peaks appear to be absent in phenols containing long saturated or unsaturated hydrocarbon chains (6, 19). This suggests that compound A might be an octaprenylphenol with a hydroxyl group in the octaprenyl side chain. Further support for this assignment comes from the peak observed at *m/e* 547 which can be attributed to an oxygen-containing fragment of the side chain resulting from benzylic cleavage. In the spectrum of 2-octaprenylphenol, this peak is absent, but an analogous fragment (not containing the hydroxyl group) is present at *m/e* 531. Thus the mass spectrum of compound A is consistent with it being a (hydroxyoctaprenyl)phenol but does not indicate the position(s) of the hydroxyl group in the side chain.

TABLE 2. *Levels of ubiquinone and the three octaprenylphenols formed by various strains of E. coli*

Strain	Level formed (μmol/g wet weight of cells)[a]			
	Ubiquinone	2-Octaprenylphenol	2-Octaprenyl-6-methoxyphenol	2-(Hydroxyoctaprenyl)-phenol
AB3311 (*ubi*$^+$)	0.15	0.01	ND[b]	ND
AN59 (*ubiB*$^-$)	ND	0.23	ND	ND
AN160 (*ubiH*$^-$)	ND	0.16	0.01	0.004

[a] Cells (about 50 g wet weight) were grown in glucose-mineral salts medium and harvested in early stationary phase, and the levels of ubiquinone and the various octaprenylphenols were measured as described in Materials and Methods.

[b] ND, Not detectable.

A comparison was made between the ultraviolet absorption spectra of compound A and various substituted phenols (Table 3) to gain further information concerning the structure of the aromatic ring of this compound. The data obtained support the identification of compound A as a (hydroxyoctaprenyl)phenol and suggest that it could be either 2- or 3-(hydroxyoctaprenyl)phenol. However, compound A gave a positive test for an *ortho*-substituted octaprenylphenol (see Materials and Methods), indicating that the compound is 2-(hydroxyoctaprenyl)phenol.

Identification of compound B as 2-octaprenylphenol. This compound was identified as the ubiquinone precursor, 2-octaprenylphenol, on the basis of its ultraviolet absorption spectrum, color with diazotized *p*-nitraniline, chromatographic properties on Silica Gel plates, and its mass spectrum. 2-Octaprenylphenol is also accumulated by *ubiB*$^-$ mutants and has been characterized previously (6).

Identification of compound C as 2-octaprenyl-6-methoxyphenol. The mass spectrum of compound C (Fig. 2) gave a molecular ion at *m/e* 668 corresponding to an octaprenylmethoxyphenol. Accurate mass measurement

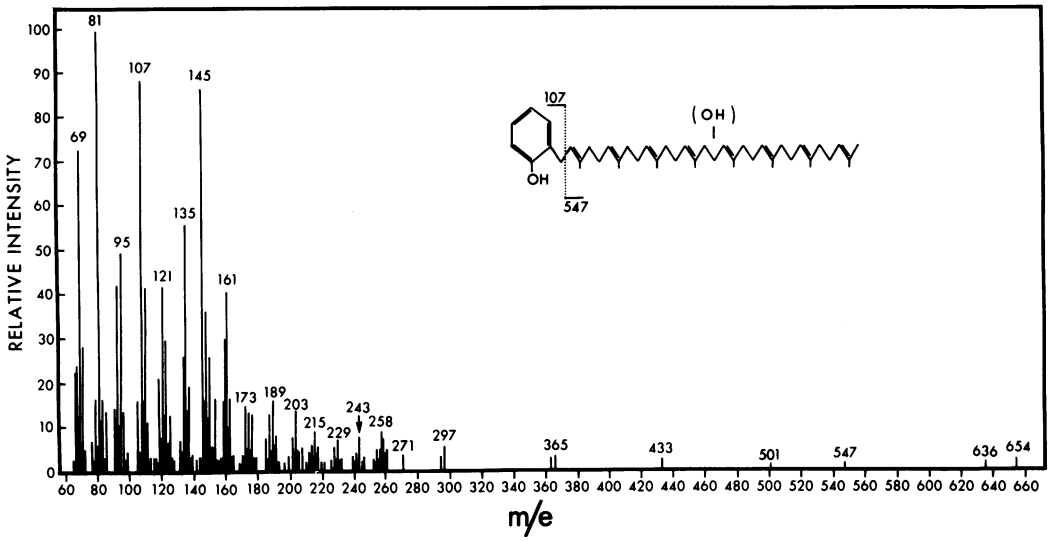

FIG. 1. *Mass spectrum of 2-(hydroxyoctaprenyl)phenol. Peaks below m/e 400 with relative intensities of <2% are not shown.*

TABLE 3. *Ultraviolet absorption spectra of compound A and model compounds*

Compound	λ Max (nm)	
	Diethyl ether	Alkaline ethanol[a]
Compound A (2-[hydroxyoctaprenyl]phenol)	274, 280S[b]	239, 289
2-Octaprenylphenol	274, 280S	239, 289
2-Methylphenol	274, 280S	239, 289
3-Methylphenol	274, 280S	239, 289
4-Methylphenol	279, 285S	
2-Allyl-4-hydroxyphenol ..	295	
2-Methyl-6-hydroxyphenol	274, 280S	251, 393
4-Allyl-6-hydroxyphenol ..	283, 290S	

[a] To 1 ml of a solution of the compound in ethanol, NaOH (6 μliters, 4 N aqueous) was added, and the spectrum was recorded immediately after mixing.

[b] S, Shoulder.

confirmed the expected composition of the molecular ion (mass found was 668.5535; $C_{47}H_{72}O_2$ requires 668.5532). The mass spectrum observed was analogous to that of 2-octaprenylphenol (6). The weak peaks present at *m/e* 599, 531, 463, 395, 327, 259, and 191 are consistent with fission of allylic carbon-carbon single bonds in the side chain with retention of charge by the fragment containing the aromatic ring. Except for the base peak at *m/e* 69, peaks corresponding to retention of charge by the alkenyl fragments in this series are much less intense. A high resolution mass spectrum showed the major peak at *m/e* 137 to be due to two components. Accurate mass measurements of these components, together with measurements of peak areas, showed that the peak at *m/e* 137 consisted of 80% of $C_8H_9O_2$ (mass 137.0596) and 20% of $C_{10}H_{17}$ (mass 137.1318), indicating that it arose predominantly from benzylic cleavage of the side chain.

The ultraviolet absorption spectrum of compound C measured in diethyl ether showed a peak at 274 nm with a shoulder at 280 nm. The spectrum was almost identical to that of 2-allyl-6-methoxyphenol but significantly different from that of 4-allyl-6-methoxyphenol (peak 281 nm, shoulder 285 nm). Thus, the ultraviolet absorption spectrum of compound C was consistent with its structure being 2-octaprenyl-6-methoxyphenol.

The nuclear magnetic resonance spectrum of compound C was measured in CCl_4 at 100 mHz (Fig. 3 and Table 4). In the aromatic region of the spectrum, a singlet (τ, 3.44 ppm) equivalent to three protons was present. Characteristic signals were also observed, indicating the presence of an hydroxyl group, a methoxyl group, and an octaprenyl side chain as the other three ring substituents (6, 10). The fact that the three aromatic protons give rise to a singlet in the spectrum is unusual for this type of ring system. The three aromatic protons of synthetic 2-nonaprenyl-6-methoxyphenol, in contrast to those of 4-nonaprenyl-6-methoxyphenol, also give a singlet (Table 4), providing further evidence that the structure of the compound is 2-octaprenyl-6-methoxyphenol.

Compounds accumulated by the other

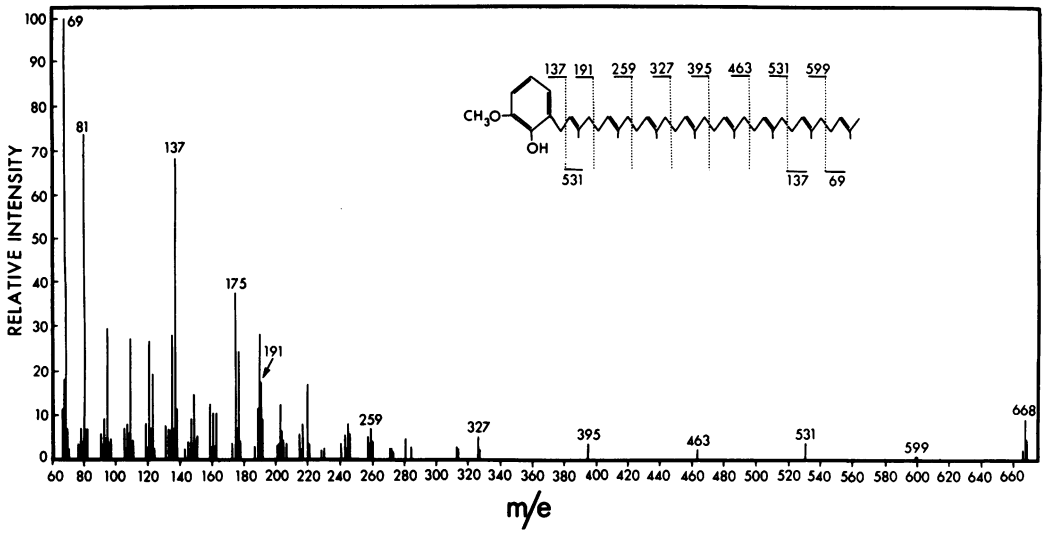

FIG. 2. *Mass spectrum of 2-octaprenyl-6-methoxyphenol. Peaks with relative intensities of <2% are not shown.*

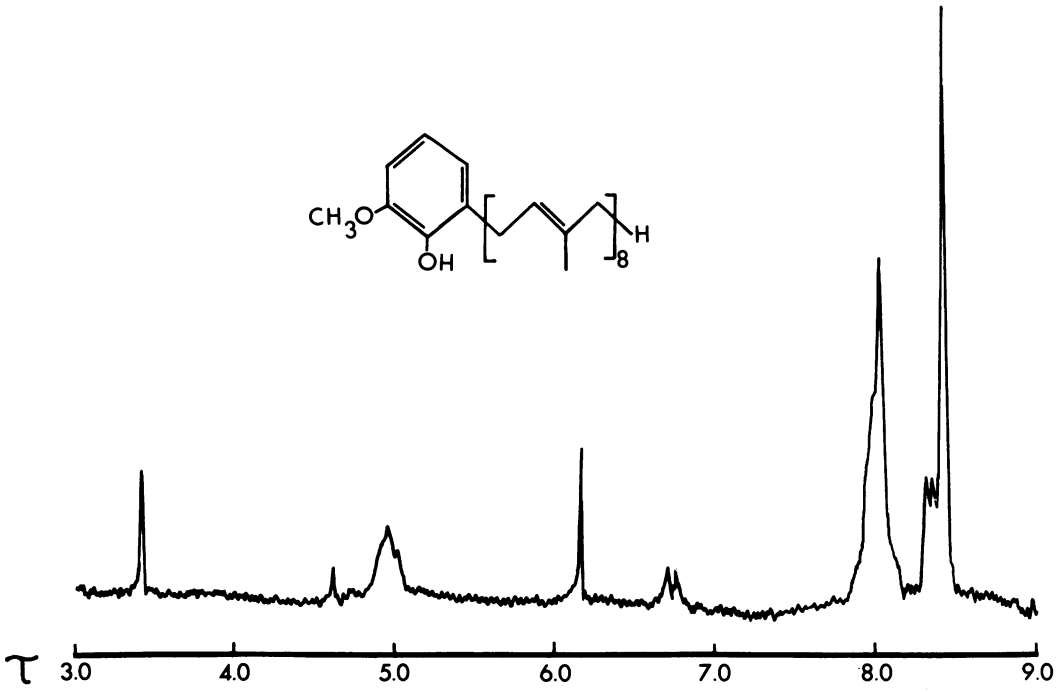

FIG. 3. *Nuclear magnetic resonance spectrum (CCl_4, 100 mHz) of 2-octaprenyl-6-methoxyphenol.*

mutants. The six other independently isolated mutants (strains AN74, AN81, AN82, AN83, AN158, and AN161) were indistinguishable from strain AN160 with respect to the compounds accumulated. All mutants accumulated small quantities of 2-(hydroxyoctaprenyl)phenol and 2-octaprenyl-6-methoxyphenol and relatively large quantities of 2-octaprenylphenol. The *ubiH*$^-$ transductants derived from these strains (see below) also showed the same pattern of accumulation, indicating that this is the phenotype of strains carrying mutations in the *ubiH* gene.

Mapping of the ubiH gene. The gene

TABLE 4. *Nuclear magnetic resonance data for compound C and related compounds (CCl_4, 100 mHz)*

Compound	Chemical shifts (ppm)					
	Aromatic	Hydroxyl	Vinyl	Methoxyl	Benzylic	Alkyl
Compound C (2-octaprenyl-6-methoxyphenol)	3.44 (s3)[a]	4.64 (s1)	4.96 (m8)	6.19 (s3)	6.75 (d2)	7.8–8.5
2-Nonaprenyl-6-methoxyphenol[b]	3.44 (s3)	4.63 (s1)	4.98 (m9)	6.19 (s3)	6.75 (d2)	7.8–8.5
4-Nonaprenyl-6-methoxyphenol[b]	3.2–3.5 (m3)	4.64 (sl)	4.95 (m9)	6.16 (s3)	6.78 (d2)	7.9–8.5

[a] s, Singlet; d, doublet; m, multiplet; the number given is the number of protons.
[b] Data for synthetic compounds (10).

carrying the mutation in strain AN83 which results in the accumulation of the three octaprenylphenols was designated *ubiH*. A streptomycin-resistant derivative of strain AN83 was converted to a female phenocopy (23) and mated with the Hfr strains AB2154, AB347, and KL16 for 1 h at 37 C. The crosses were then interrupted, and selection was made for *ubi*$^+$ recombinants by using succinate as sole carbon source. Strains AB2154 and KL16 transfer their chromosomes from min 74 and min 55, respectively, with an anti-clockwise order of gene transfer, and strain AB347 transfers from min 60 in a clockwise direction. *ubi*$^+$ recombinants were obtained in the case of the cross with strain AB2154 but not in the case of crosses with strains AB347 or KL16, suggesting that the *ubiH* gene was located on the chromosome between min 55 and min 60. To further locate the *ubiH* gene, co-transduction was attempted with the *lysB* and *serA* genes which are located at about min 55 and 56.5, respectively (24).

The *ubiH* gene was found to be co-transducible with *serA* at a frequency of 72% and with *lysB* at 2% (Table 5), locating it at about min 56.5 on the chromosome map compiled by Taylor (24).

Mapping of the genes affected in the other strains accumulating the three octaphenylphenols. Transduction crosses were carried out with each of the other strains which accumulated the three octaprenylphenols as donors and with strain AT2475 (*serA6*) as recipient. The mutations carried by each of the strains were co-transducible with *serA6*, and all co-transduction frequencies were in the range of 63 to 85% (average 75%). This suggested that each of these strains might be affected in the same gene as AN83, and this was tested further by two-factor transduction crosses between the mutants (Table 6). The low frequency of recombination observed in transduction crosses involving the mutant alleles is consistent with each of the mutations carried by these strains being in the *ubiH* gene.

Pathway for ubiquinone biosynthesis in E. coli. Since the *ubiH* mutants accumulate 2-octaprenyl-6-methoxyphenol, it seems likely that this compound is a ubiquinone precursor in *E. coli* and that the *ubiH* mutants are blocked in its conversion to 2-octaprenyl-6-

TABLE 5. *Transduction data for the ubiH gene*

Donor strain	Recipient strain	Marker selected	No. of transductants carrying unselected donor trait
AN83 (*ubiH424*)	AT2475 (*serA6*)	*serA*$^+$	58/81 (72%) *ubiH*$^-$
AT713 (*lysB10*)	AN172 (*ubiH424*)	*ubiH*$^+$	2/80 (2%) *lysB*$^-$

TABLE 6. *Transduction crosses between ubiH$^-$ mutants*

Donor[a] strain	No. of *leu*$^+$ transductants with AB1515 (*leu*$^-$) as recipient	No. of *ubi*$^+$ transductants with AN83 (*ubiH424*) as recipient	No. of *ubi*$^+$ as percentage of wild-type frequency
AB2826 (*leu*$^+$, *ubi*$^+$)	372	302	100
AN74 (*leu*$^+$, *ubiH425*)	222	0	<1
AN81 (*leu*$^+$, *ubiH426*)	423	0	<1
AN82 (*leu*$^+$, *ubiH427*)	724	0	<1
AN158 (*leu*$^+$, *ubiH428*)	479	4	<1
AN160 (*leu*$^+$, *ubiH429*)	492	0	<1
AN161 (*leu*$^+$, *ubiH430*)	188	1	<1

[a] A standard quantity of phage was used to transduce both recipients under the same conditions so that the number of *ubi*$^+$ transductants could be related to the number of *leu*$^+$ transductants.

methoxy-1,4-benzoquinone (see Fig. 6). Studies involving other ubiquinone-deficient mutants have indicated that 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone is an intermediate in the conversion of 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone to ubiquinone in *E. coli* (23). By analogy with this conversion, one would predict the existence of 2-octaprenyl-6-hydroxyphenol as an intermediate in the synthesis of 2-octaprenyl-6-methoxyphenol from 2-octaprenylphenol (Fig. 4). The hypothetical intermediate, 2-polyprenyl-6-hydroxyphenol, has been included in a previous scheme for ubiquinone biosynthesis proposed by Friis et al. (12).

Strain AN164 (*metB*$^-$, *aroB*$^-$) was used in an attempt to test whether 2-octaprenyl-6-hydroxyphenol exists as a free ubiquinone intermediate in *E. coli*. This strain is blocked in the common pathway of aromatic biosynthesis prior to chorismate and is thus unable to synthesize the quinone rings of ubiquinone and menaquinone. However, it grows satisfactorily if the other aromatic compounds derived from chorismate are provided in the growth medium (14). Cells can therefore be grown which lack ubiquinone but which possess all the enzymes required for the synthesis of ubiquinone from 4-hydroxybenzoate. The metabolism of added 4-hydroxybenzoate can then be followed in nongrowing suspensions of these cells.

The methyl groups of the quinone ring of ubiquinone have been shown to be derived from methionine in *E. coli* (16). Since 2-octaprenyl-6-methoxyphenol appears to be the first intermediate in ubiquinone synthesis possessing a methyl group (see Fig. 6), it would be expected that in the absence of methionine cells should accumulate 2-octaprenyl-6-hydroxyphenol if it exists as a free intermediate. This compound should have an ultraviolet absorption spectrum and color with the *p*-nitraniline spray almost identical to those of 2-methyl-6-hydroxyphenol and would be expected to run with an R_f of about 0.2 using solvent A.

Cells of strain AN164 were grown under conditions where the quantities of tryptophan and methionine limited their growth and where they were unable to synthesize ubiquinone or menaquinone (see Materials and Methods). They were then incubated for 6 h at 37 C in unsupplemented glucose-mineral salts medium together with 4-hydroxybenzoate, in the presence and absence of methionine. The cells incubated with methionine formed ubiquinone (0.07 μmol/g wet weight) and 2-octaprenylphenol (0.10 μmol/g wet weight). The cells incubated in the absence of methionine formed higher levels of 2-octaprenylphenol (0.16 μmol/g wet weight), but, as expected, no ubiquinone was formed. In addition, no 2-(hydroxyoctaprenyl)phenol was formed, and no compound with the properties of 2-octaprenyl-6-hydroxyphenol was detected. In a control experiment, it was shown that a good recovery of 2-methyl-6-hydroxyphenol was obtained when this compound was submitted to the extraction procedure used. Thus, no evidence was obtained for the existence of 2-octaprenyl-6-hydroxyphenol as a free intermediate in the conversion of 2-octaprenylphenol to 2-octaprenyl-6-methoxyphenol.

DISCUSSION

The studies of the *ubiH* mutants indicate that a mutation in the *ubiH* gene can cause both the inability to form ubiquinone and the accumulation of 2-octaprenylphenol, 2-octaprenyl-6-methoxyphenol, and 2-(hydroxyoctaprenyl)phenol. 2-Octaprenylphenol has been isolated previously and characterized from *ubiB* mutants of *E. coli*, and strong evidence was obtained that it is a precursor of ubiquinone in this organism (6). The isolation of 2-octaprenyl-6-methoxyphenol from the *ubiH* mutants represents the first characterization of this compound from *E. coli*. It seems likely that 2-octaprenyl-6-methoxyphenol is also a ubiquinone precursor and that the *ubiH* mutants are blocked in the conversion of this compound to 2-octaprenyl-6-methoxy-1,4-benzoquinone. The reason why low levels of 2-octaprenyl-6-methoxyphenol and relatively high levels of 2-octaprenylphenol are accumulated by *ubiH* mutants is not known. 2-Polyprenyl-6-methoxyphenol has previously been isolated and characterized from *Rhodospirillum rubrum* (20), rat liver (18), and *Pseudomonas ovalis* (28). The relevance of the accumulation of 2-(hydroxyoctaprenyl)phenol to the biosynthesis of ubiquinone is not clear at present.

No evidence was obtained for the accumulation of 2-octaprenyl-6-hydroxyphenol by the *ubiH*$^-$ strains, and this compound was not accumulated in the experiment involving methionine limitation. We therefore conclude that 2-octaprenyl-6-hydroxyphenol may not exist as a free intermediate in the biosynthesis of ubiquinone.

The *ubiH* gene is not closely linked to any of the other six *ubi* genes previously mapped. Recently, the gene (*ubiC*) which codes for the

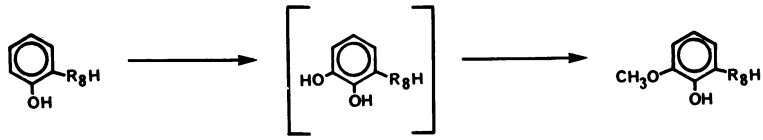

FIG. 4. *The conversion of 2-octaprenylphenol to 2-octaprenyl-6-methoxyphenol via the hypothetical intermediate 2-octaprenyl-6-hydroxyphenol. R_8H denotes the octaprenyl side chain.*

enzyme catalyzing the conversion of chorismate to 4-hydroxybenzoate has been identified and shown to be located close to the *ubiA* gene at minute 79 (J. Lawrence, unpublished results). Thus, the eight genes that have been identified as being involved in ubiquinone biosynthesis are located on the *E. coli* chromosome as two clusters of closely linked genes at min 75 and min 79, with the remaining genes being at min 16, 42, and 56 (Fig. 5). These eight genes are probably the structural genes coding for the enzymes concerned with ubiquinone biosynthesis.

The isolation and characterization of the ubiquinone precursors accumulated by the *ubiH* mutants and by the other classes of *ubi* mutants studied previously (6, 23, 30, 31) enable a pathway for ubiquinone biosynthesis in *E. coli* to be formulated (Fig. 6). Each intermediate in the sequence has been adequately characterized. Biochemical studies using cell extracts are required to confirm the proposed compounds as intermediates and to gain an understanding of the mechanisms of the individual reactions, most of which are membrane associated. The first three reactions of the pathway have been demonstrated in cell extracts of *E. coli* by using mutants to accumulate the appropriate substrates (6, 14, 30; J. Lawrence, unpublished results), but the remainder of the reactions have yet to be examined. The results of the present experiments with cell suspensions indicate that nongrowing cells of *E. coli* can form good levels of ubiquinone, and it seems likely that the synthesis of ubiquinone may not be dependent on membrane synthesis.

Mutants have been obtained blocked in every step of the proposed pathway and, with the exception of the *ubiD*$^-$ strain reported previously (6), are completely blocked and unable to form ubiquinone. Recent studies with other *ubiD* mutants (R. A. Leppik, unpublished results) have indicated that a general characteristic of *ubiD*$^-$ strains is that they form about 20% of the normal level of ubiquinone. These strains appear to form 2-octaprenylphenol from 3-octaprenyl-4-hydroxybenzoate by an alternative reaction which requires the accumulation

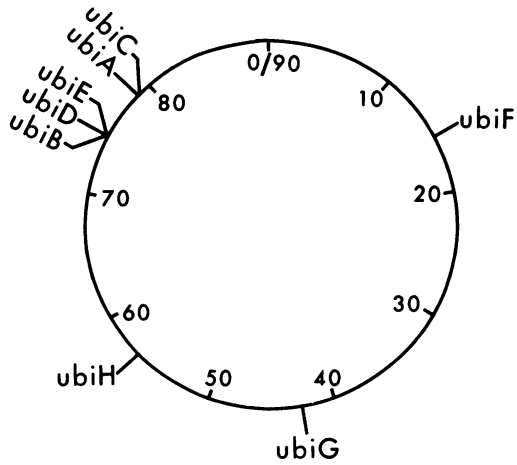

FIG. 5. *Chromosomal map of E. coli showing the approximate positions of the genes that are known to be concerned with ubiquinone biosynthesis.*

of appreciable quantities of 3-octaprenyl-4-hydroxybenzoate. This reaction is probably of little significance in a wild-type strain. The above results suggest that there is only one major pathway leading to ubiquinone in *E. coli* K-12 and that the 4-hydroxybenzoate required for the pathway is solely derived from chorismate. The ubiquinone pathway provides a model of a membrane-associated biosynthetic pathway, and further studies should provide a better understanding of the interaction of the various enzymes and their substrates with the cell membrane.

Apart from the work with *E. coli*, the biosynthesis of ubiquinone in a number of other species of bacteria, in fungi, and in higher organisms has also been studied. In general, the approach used has been to detect and identify polyisoprenoid compounds, which might be ubiquinone precursors, in extracts of lipids from normal organisms. This method relies on the steady-state level of an intermediate being sufficiently high to allow its isolation and identification. Unfortunately, few intermediates in a pathway may be present in such quantities, and in some cases none of the intermediates may be detectable (17, 26, 28, 29). Nevertheless, a number of polyprenylphe-

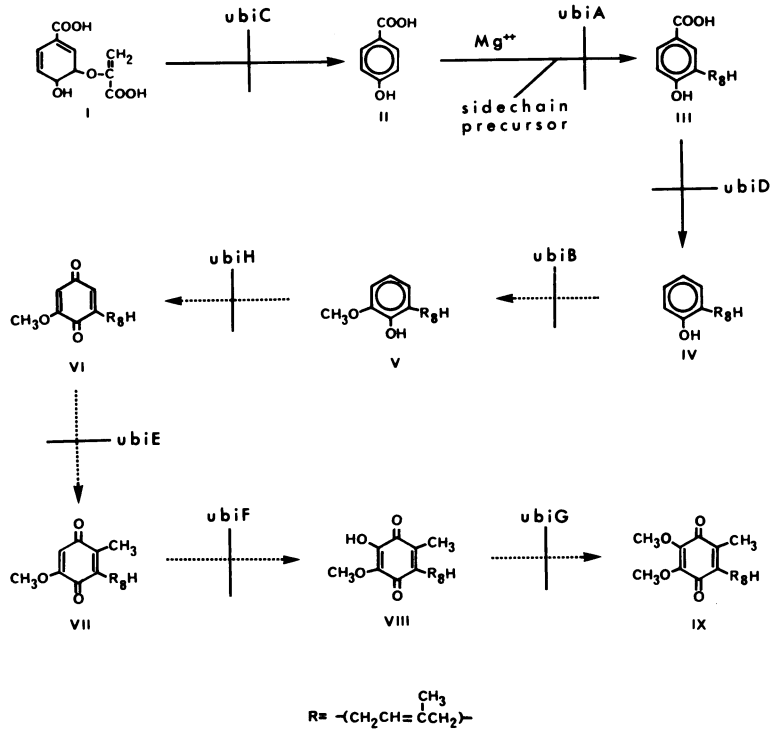

FIG. 6. *The pathway for ubiquinone biosynthesis in E. coli established by using ubiquinone-deficient mutants. Reactions demonstrated in cell extracts are shown with solid arrows. The reactions affected by mutations in the various ubi genes are also shown. I, chorismic acid; II, 4-hydroxybenzoic acid; III, 3-octaprenyl-4-hydroxybenzoic acid; IV, 2-octaprenylphenol; V, 2-octaprenyl-6-methoxyphenol; VI, 2-octaprenyl-6-methoxy-1,4-benzoquinone; VII, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone; VIII, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone; IX, ubiquinone.*

nols and quinones have been isolated and characterized. These comprise 2-polyprenyl-6-methoxyphenol and 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone, (*R. rubrum* [12], *P. ovalis* [15, 28], rat liver [18, 27]), 2-polyprenylphenol (*P. ovalis* [15], *R. rubrum* [21]), and 2-polyprenyl-6-methoxy-1,4-benzoquinone (*P. ovalis* [28]). On the basis of these and other studies, several distinct pathways for ubiquinone biosynthesis have been proposed (12, 18, 28), but it has not been possible to establish adequately a complete pathway for any one organism. The first pathway proposed, based on the isolation of three polyprenyl precursors from *R. rubrum* (12), is very similar to the pathway given in Fig. 6 (with the exception of the presence of 2-polyprenyl-6-hydroxyphenol) and has been a valuable model for subsequent studies in a variety of organisms.

The pathway given in Fig. 6 which is based solely on work with *E. coli* represents the best documented pathway for the biosynthesis of ubiquinone in any organism. The fact that several of the intermediates given in Fig. 6 have been isolated from other bacteria and rat liver suggests that the biosynthesis of ubiquinone from 4-hydroxybenzoate may be essentially the same in all organisms and that results obtained with *E. coli* may prove to be widely applicable.

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