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Mutants of *Escherichia coli* K-12 Blocked in the Final Reaction of Ubiquinone Biosynthesis: Characterization and Genetic Analysis

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The ubiquinone precursor 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone was isolated from two ubiquinone-deficient mutants of *Escherichia coli* and identified by nuclear magnetic resonance and mass spectrometry. The results of genetic analysis of the mutants indicate that each mutant carries a mutation in a gene designated *ubiG* which was located, by cotransduction with the *nalA* and *glpT* genes, at minute 42 on the *E. coli* chromosome.

The examination of mutants of *Escherichia coli* which are unable to form ubiquinone has enabled the isolation and characterization of a number of intermediates in ubiquinone biosynthesis (3, 14). The mutants form sufficient amounts of the compounds to allow their detailed chemical characterization. This is often difficult or impossible with the small quantities of compounds obtained by other methods of investigation (see 14). Four classes of mutants accumulating the ubiquinone precursors 3-octaprenyl-4-hydroxybenzoate, 2-octaprenylphenol, 2-octaprenyl-6-methoxy-1,4-benzoquinone, and 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone have been described previously, and the four genes affected in these mutants have been located on the *E. coli* chromosome (3, 14). Three of them (*ubiB*, *ubiD*, and *ubiE*) are closely linked at minute 75, and the other gene (*ubiF*) is located at minute 16.

The present paper describes the characterization of two new mutants, each blocked in the final reaction of ubiquinone biosynthesis.

MATERIALS AND METHODS

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

Media. The minimal medium used was the mineral-salts medium 56 slightly modified from that described by Monod et al. (8) and containing (per liter): K_2HPO_4, 10.6 g; $NaH_2PO_4 \cdot 2H_2O$, 6.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 2 g; $Ca(NO_3)_2$, 10 mg; and $FeSO_4 \cdot 7H_2O$, 0.5 mg. Supplements were added as sterile solutions to give the following final concentrations: D-glucose, 30 mM; sodium succinate, 30 mM; DL-α-sodium glycerophosphate, 20 mM; L-leucine, 0.8 mM; L-threonine, 0.7 mM; L-isoleucine, 0.3 mM; L-valine, 0.3 mM; L-arginine-HCl, 0.8 mM; L-proline, 1.5 mM; L-tryptophan, 0.2 mM; L-histidine-HCl, 0.15 mM; L-methionine, 0.2 mM; adenine-HCl, 0.15 mM; and thiamine-HCl, 1 μM.

Conjugation and transduction experiments. The technique for conjugation experiments was based on that described by Taylor and Thoman (11), and transduction experiments, in which the generalized transducing phage P1kc was used, were carried out as described by Pittard (9).

Isolation of strains resistant to nalidixic acid. Nalidixic acid-resistant derivatives of various strains were isolated by spreading cells from an overnight culture in nutrient broth onto nutrient agar containing nalidixic acid at a final concentration of 50 μg/ml. Nalidixic acid was used at this concentration to isolate resistant strains carrying mutations in the *nalA* gene (see 6).

Transduction of nalidixic acid resistance. Sensitivity to nalidixic acid is dominant over resistance (6); therefore, a period of growth to allow phenotypic expression to occur was given prior to exposure to nalidixic acid. Samples of transduced recipient cells were plated in 0.8% agar onto minimal medium containing 10 mM sodium citrate to reduce further phage infection. The plates were incubated for 4 hr at 37 C prior to the addition of nalidixic acid in agar to give a final concentration of 50 μg/ml.

Conversion of male strains to "phenocopy females." Strains were grown overnight in nutrient broth in flasks shaken at 37 C, and the cells were collected by centrifugation, resuspended in unsupplemented medium 56, and shaken at 37 C for approximately 5 hr. Male strains treated in this way could be mated satisfactorily with Hfr males.

Isolation and characterization of mutant strains. The methods used for the isolation of ubi-

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

Strain[a]	Sex	Relevant genetic loci[b]	Other information
AB259	Hfr		Hfr Hayes
AB311	Hfr	*thr-1, leu-6*	
AB3282	F⁻	*ilvC7, argE3, leu-351, proA2, trp⁻, his-4, str-704*	
AB3311	Hfr	*metB⁻*	Hfr Reeves I
AN86	Hfr	*metB⁻, ubiG422*	Isolated from AB3311 after treatment with MNNG[c]
AN145	F⁺	*ilvC7, argE3, leu-351, proA2, trp⁻, ubiG422, str-704*	
AN151	Hfr	*metB⁻, ubiG423*	Isolated from AB3311 after treatment with MNNG[c]
AN189	Hfr	*metB⁻, ubiG422, nalA^R*	Spontaneous mutant from AN86
AN190	Hfr	*metB⁻, ubiG423, nalA^R*	Spontaneous mutant from AN151
MH5	Hfr	*nalA^R*	Hane and Wood (6)
KL98	Hfr		
221	Hfr	*glpT⁻*	Cozzarelli et al. (4)
AT2092 (16.1)	F⁻	*arg⁻, his⁻, purF⁻, aroF⁻*	

[a] Strain MH5 was kindly provided by M. W. Hane, strain 221 by E. C. C. Lin, strain AT2092 (16.1) by K. D. Brown, and strain KL98 by B. Low.

[b] Genetic nomenclature is that used by Taylor (10).

[c] MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

quinone-deficient mutants and for the extraction and chromatography of the quinones were described previously (2, 3).

Preparation of silica gel thin-layer chromatography plates containing acid. Silica gel plates containing two different concentrations of H_2SO_4 were used. Merck silica gel G (50 g) was suspended in 100 ml of water containing either 0.12 ml (0.2%, w/v) or 0.4 ml (0.7%, w/v) of concentrated H_2SO_4, and plates (20 cm by 20 cm) were poured. The plates were dried at 110 C for 2 hr.

Isolation of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. Strain AN151 was grown in 1-liter quantities in 2-liter flasks shaken at 37 C. The cells were harvested and extracted as described previously (3). The light petroleum solution obtained was then chromatographed by use of 0.7% H_2SO_4-silica gel plates, with chloroform-light petroleum (70:30, v/v) as solvent. The new compound ran as an orange band with an R_F of 0.7. After elution with diethyl ether, the quinone was further purified by chromatographing twice on 0.7% acid-silica gel plates (R_F, 0.7) and twice on 0.2% acid-silica gel plates (R_F, 0.5) with ethyl acetate-hexane (25:75, v/v) as solvent. The compound was eluted with diethyl ether, and the eluate was taken to dryness on a rotary evaporator and dried at −20 C in a vacuum desiccator over P_2O_5 and paraffin wax. The quantity of purified compound from 380 g (wet weight) of cells was 4.5 mg.

Spectroscopy. Mass spectra were obtained with an AEI MS9 double-focus mass spectrometer. Nuclear magnetic resonance spectra were obtained with a Varian HA100 spectrometer with tetramethylsilane as internal standard. Ultraviolet spectra were obtained with a Cary 15 spectrophotometer.

RESULTS

A large number of ubiquinone-deficient mutants of *E. coli* have been isolated from strain AB3311 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by screening for strains unable to grow on succinate but able to grow on glucose as sole carbon source, and then selecting from this class those mutants forming little or no ubiquinone. The characterization and genetic analysis of four groups of mutants accumulating different ubiquinone precursors have been reported previously (3, 14). Two strains, AN86 and AN151, accumulating another precursor of ubiquinone have now been examined in detail.

Detection and identification of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone accumulated by strain AN151. Cells of strain AN151 (3 g, wet weight) were extracted, and the lipid extract was chromatographed on a silica gel plate with chloroform-light petroleum (70:30, v/v) as solvent. A normal level of menaquinone was observed (R_F, 0.7), whereas the quantity of ubiquinone (R_F, 0.5) present was about 5% of the normal level. Two compounds accumulated by the mutant strain were detected. One of these was identified as 2-octaprenylphenol (R_F, 0.6), which has been described in detail previously (3). This compound was estimated to be present in an amount equivalent to about 60% of the normal amount of ubiquinone. The second compound

appeared as a narrow purple band (R_F, 0.1) which, after elution from the gel with acidified diethyl ether, gave a yellow solution with an ultraviolet absorption spectrum showing a peak at 270 nm. This peak was very similar to that of ubiquinone except that it was less symmetrical. When ethanol was used as solvent, the peak was shifted to 275 nm and became more symmetrical. The spectrum of the new compound and the spectral changes observed after the addition of sodium borohydride (Fig. 1) suggested that it was a substituted 1:4 benzoquinone closely related to ubiquinone. The new compound was formed at about 30% of the normal level of ubiquinone, assuming it had an extinction coefficient equal to that of ubiquinone. The behavior of the compound on chromatography, including its purple color on silica gel, suggested the presence of a hydroxyl substituent on the benzoquinone ring.

Initial attempts to purify the compound on silica gel plates gave low recoveries. It was found that good recoveries were obtained when the compound was chromatographed on acid-silica gel plates, where it ran as an orange band with an R_F close to that of ubiquinone.

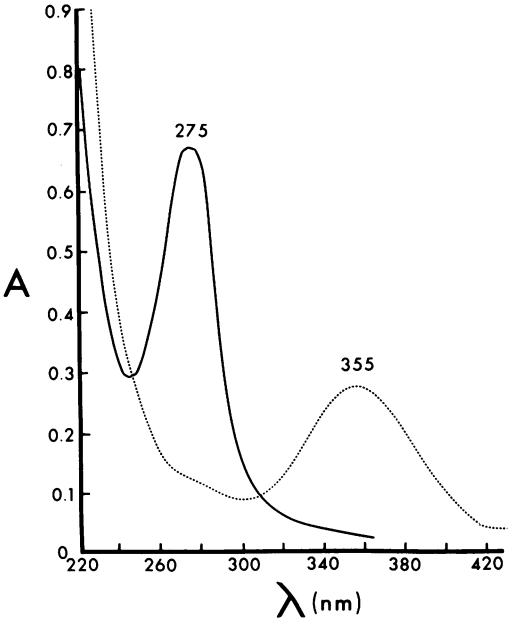

FIG. 1. *Ultraviolet absorption spectra of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone in ethanol before and after reduction with sodium borohydride. Solid line, quinone; broken line, quinol. The spectrum in the presence of sodium borohydride was unstable and was recorded when the absorption at 275 nm reached its lowest value.*

Strain AN151 was used to accumulate a sufficient amount of the new compound to allow purification as described in Materials and Methods. The compound was an orange oil at room temperature and was identified as 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone by nuclear magnetic resonance and mass spectrometry.

The nuclear magnetic resonance spectrum of the compound (Fig. 2) was measured at 100 MHz with deuteropyridine as solvent. The spectrum shows characteristic signals of appropriate intensity for an octaprenyl side chain attached to a 1,4-benzoquinone nucleus (3). The only other signals present are attributable to a ring methyl group and ring methoxyl group, indicating the presence of an additional ring substituent whose signal is lost, presumably owing to exchange. The chemical shifts of the signals due to the ring substituents are consistent with the structure 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone or the corresponding -5-methoxy-6-hydroxy isomer. A comparison of the chemical shifts obtained for the present compound with published data for synthetic model compounds (Table 2) indicates that the ubiquinone precursor has the structure 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone.

The mass spectrum of the ubiquinone precursor (Fig. 3) showed the expected molecular ion at *m/e* 712, together with a P + 2 peak at *m/e* 714 characteristic of quinones (1). The accurate mass of the molecular ion was found to be 712.546982 ($C_{48}H_{72}O_4$ requires 712.543074). The base peak in the spectrum is at *m/e* 221 and can be attributed to the formation of a stable ion in which all but four of the carbon atoms of the isoprenoid side chain have been lost (7). The peak at P + 2, characteristic of the quinol, increased markedly with time, whereas the peak due to the quinone decreased in intensity. Concurrently with this change, the intensity of the base peak at *m/e* 221 decreased and the peak at *m/e* 183 increased. The ion at *m/e* 183 ($C_9H_{11}O_4$) can be attributed to a tropylium ion derived from the quinol in which all but one of the carbon atoms of the side chain have been lost.

Genetic analysis of strains AN86 and AN151. In addition to strain AN151, an independently isolated strain (AN86) was also shown to accumulate 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. The gene carrying the mutation present in strain AN86 which results in the accumulation of the above compound was designated *ubiG*. Strain AN86 (Hfr, *ubiG*$^-$) was mated with AB3282 (♀,

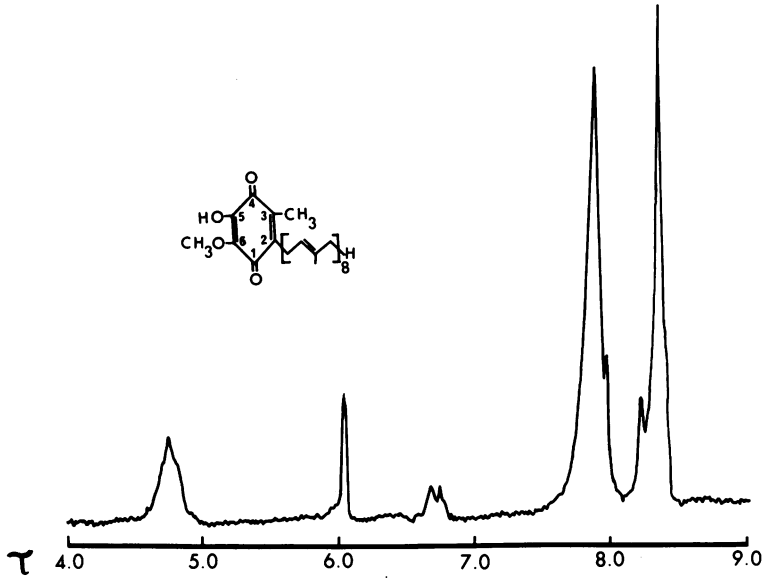

FIG. 2. *Nuclear magnetic resonance spectrum (deuteropyridine, 100 MHz) of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone.*

TABLE 2. *Nuclear magnetic resonance data for the ubiquinone precursor and synthetic model compounds (deuteropyridine, 100 MHz)*

Compound	Chemical shift (ppm)				
	Olefinic	Methoxyl	Ring methylene	Ring methyl	Alkyl
2-Octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone	4.75	6.05	6.69	7.97	7.88, 8.22, 8.34, 8.40
2-Decaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone[a]	4.75	6.04	6.69	7.98	7.89, 8.22, 8.36, 8.41
2-Decaprenyl-3-methyl-5-methoxy-6-hydroxy-1,4-benzoquinone[a]	4.74	6.02	6.76	—[b]	7.88, 8.22, 8.35, 8.41

[a] Data from Wilczynski et al. (13).
[b] Ring methyl signal obscured by broad alkyl signal.

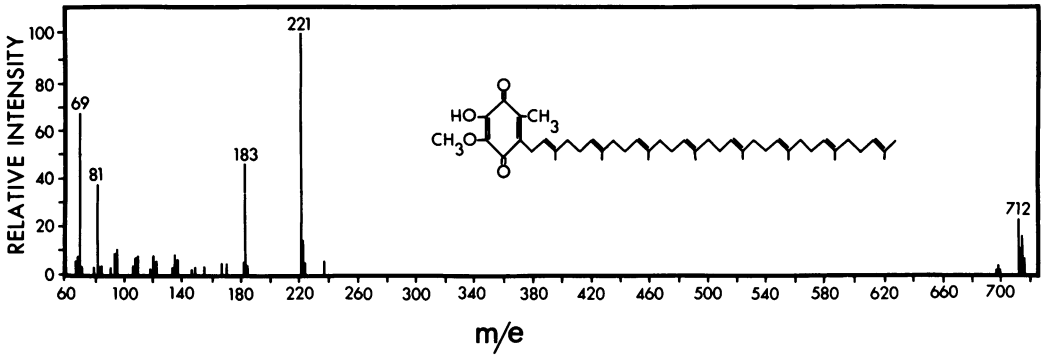

FIG. 3. *Mass spectrum of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. Peaks with relative intensities of <2% are not shown.*

ilv$^-$, *leu*$^-$, *trp*$^-$, *his*$^-$); after 2 hr at 37 C the mating was interrupted, and *ilv*$^+$, *leu*$^+$, *trp*$^+$, and *his*$^+$ recombinants were selected and tested for their ability to grow on succinate as sole carbon source. All of the recombinants unable to grow on succinate were *his*$^+$, and one of these strains (AN145) was shown, by extraction and chromatography, to be unable to synthesize ubiquinone and to accumulate 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, confirming that it had received the *ubiG*$^-$ allele. Strain AN86 transfers its chromosome from minute 74 with a clockwise order of gene transfer, and since only the *his*$^+$ recombinants had received the *ubiG*$^-$ allele it appeared that the *ubiG* gene might be located near *his* (minute 39). Strain AN145 (*ubiG*$^-$, *trp*$^-$) was converted to a phenocopy female and used as recipient for matings with the Hfr males AB311 and KL98. The *ubiG* gene was not transferred as an early marker by AB311, although normal numbers of *trp*$^+$ recombinants were obtained. The *ubiG* gene was transferred as an early marker by KL98, and a time of entry of about 10 min was obtained. Since AB311 and KL98 transfer their chromosomes with origins at minute 39 and minute 44, respectively, and the order of gene transfer is anticlockwise in both cases, the *ubiG* gene must be located on the chromosome between minute 39 and minute 44, probably at about minute 42.

To locate the *ubiG* gene more precisely, attempts were made to detect cotransduction between the *ubiG* gene and the *glpT*, *nalA*, and *purF* genes which had been located previously in the region between minute 42 and minute 44 (4, 6, 12). The results of transduction experiments with the generalized transducing phage P1kc are given in Table 3. The *ubiG* gene was cotransducible with *nalA* at a frequency of 98% and with *glpT* at 74%. No cotransduction was detected between the *ubiG* gene and *purF*. The cotransduction figures indicate that the *ubiG* gene is situated on the chromosome close to the *glpT* and *nalA* genes, between minute 42 and minute 43 according to the map of Taylor (10).

The mutation in the other ubiquinone-deficient mutant (AN151) was found to be cotransducible with *nalA* at a frequency of 95% (Table 3). This suggests that both mutants carry mutations in the *ubiG* gene.

Both of the *ubiG*$^-$ mutants (AN86 and AN151), and also the *ubiG*$^-$ transductants derived from these strains (AN189 and AN190), accumulate 2-octaprenylphenol as well as 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. This suggests that the accumulation of 2-octaprenylphenol is a characteristic of *ubiG*$^-$ strains and is not due to the presence of two mutations affecting ubiquinone synthesis.

DISCUSSION

The isolation of strains AN86 and AN151 has allowed the isolation and chemical characterization of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. This compound has not been characterized previously, although some evidence for the formation of the corresponding multiprenyl compound was obtained as a result of experiments on the metabolism of ^{14}C-4-hydroxybenzoate by *Rhodospirillum rubrum* (5).

The *ubiG* gene is located at minute 42 on the *E. coli* chromosome, well separated from the clustered *ubi* genes (B, D, and E) at minute 75 and the *ubiF* gene which is located at minute 16. The high frequency of cotransduction of the *nalA* gene and the *ubiG* gene suggests that these two genes may be contiguous. Although the product of the *nalA* gene is

TABLE 3. *Transduction between the ubiG gene and the nalA, glpT, and purF genes*

Donor strain	Recipient strain	Marker selected	No. of transductants carrying the unselected marker
AN86 *nalA*^{R} (*ubiG422*, *nalA*^{R})	AB259 (*ubiG*$^+$, *nalA*^{S})	*nalA*^{R}	79/80 *ubiG*$^-$ (99%)
AN151 *nalA*^{R} (*ubiG423*, *nalA*^{R})	AB259 (*ubiG*$^+$, *nalA*^{S})	*nalA*^{R}	76/80 *ubiG*$^-$ (95%)
221 (*ubiG*$^+$, *glpT*$^-$)	AN145 (*ubiG422*, *glpT*$^+$)	*ubiG*$^+$	28/38 *glpT*$^-$ (74%)
MH5 (*ubiG*$^+$, *nalA*^{R})	AN145 (*ubiG422*, *nalA*^{S})	*ubiG*$^+$	27/28 *nalA*^{R} (97%)
MH5 (*glpT*$^+$, *nalA*^{R})	221 (*glpT*$^-$, *nalA*^{S})	*nalA*^{R}	28/45 *glpT*$^+$ (62%)
		glpT$^+$	17/27 *nalA*^{R} (63%)
AN86 (*purF*$^+$, *ubiG422*)	AT2092 (16.1) (*purF*$^-$, *ubiG*$^+$)	*purF*$^+$	0/80 *ubiG*$^-$ (<1%)

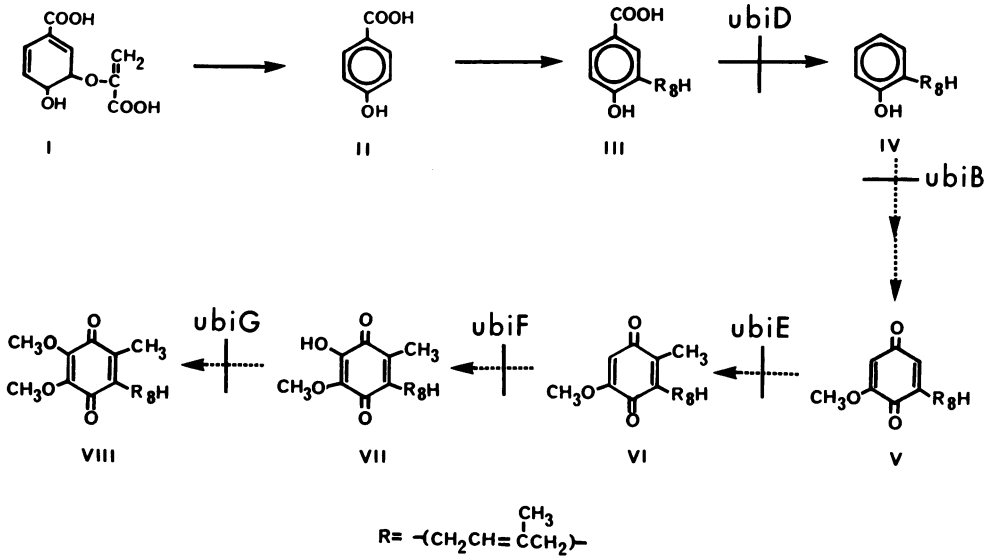

FIG. 4. *Intermediates in ubiquinone biosynthesis in E. coli established with mutants or cell-free extracts, or both. The metabolic blocks caused by mutations in the ubiB, ubiD, ubiE, ubiF, and ubiG genes are indicated. Reactions demonstrated with cell-free extracts are shown with solid arrows. The compounds are as follows: I, chorismic acid; II, 4-hydroxybenzoic acid; III, 3-octaprenyl-4-hydroxybenzoic acid; IV, 2-octaprenylphenol; V, 2-octaprenyl-6-methoxy-1,4-benzoquinone; VI, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone; VII, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone; VIII, ubiquinone-8.*

not known, the two genes appear to be functionally unrelated, because *ubiG*$^-$ strains are sensitive to nalidixic acid and strain MH5 (*nalA*^{R}) synthesizes normal amounts of ubiquinone.

The intermediates in ubiquinone biosynthesis so far established by work with ubiquinone-deficient mutants of *E. coli* K-12 are depicted in Fig. 4. Classes of mutants which have not yet been described are being examined to elucidate further the genetics and biochemistry of ubiquinone biosynthesis.

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