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Biosynthesis of Menaquinone (Vitamin K_2) and Ubiquinone (Coenzyme Q)

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INTRODUCTION

Facultatively anaerobic Gram-negative bacteria including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium contain the isoprenoid quinones of the benzene and naphthalene series. The structures of these quinones are shown in Fig. 1. According to the IUPAC-IUB recommendations (65), the benzoquinones are termed ubiquinones (Q-n) (structure I in Fig. 1) and the naphthoquinones are termed either menaquinones (MK-n) (structure II in Fig. 1) or demethylmenaquinones (DMK-n) (structure III in Fig. 1). The n refers to the number of prenyl units present in the side chain. It should be pointed out that while MK is considered a vitamin (vitamin K_2), Q is not, due to the fact that vitamin-K is an essential nutrient (can not be synthesized) by mammals, while Q is not an essential nutrient since it can be synthesized from the aromatic amino acid tyrosine.

The major quinones in *E. coli* are Q-8, MK-8, and DMK-8; minor amounts of Q-1 to Q-7, Q-9 and MK-6, MK-7, MK-9, and DMK-7 may also be present (23). The prenyl side chains have all-*trans* configuration (9). In contrast to the extensive investigations on the quinone composition and biosynthesis in *E. coli*, *S. enterica* has been studied to a lesser extent. These organisms neither have quinones that have one or more of the prenyl residues of the side chain reduced nor MK with more than one methyl group. Methods for the extraction, purification, identification and analysis of the quinones have been reviewed extensively (9, 22, 27, 33, 46, 59, 83, 85, 96, 100–102, 113, 127, 128, 130, 144, 157, 160, 170).

Since the last edition of *E. coli/Salmonella*, the following reviews on the subject have appeared. Reviews on the reaction mechanisms of various enzymes involved in MK and Q biosynthetic pathways have been published by Begley (6) and Meganathan (103). Two short reviews on Q biosynthesis have appeared (107, 152). In most of these reviews, the work on *E. coli* and to a lesser extent on *Salmonella* predominated due to the ease with which these organisms can be manipulated. However, due to advances in technology, it has become a reality that work on other bacteria can be carried out with ease.

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Hence, driven by economic and chemotherapeutic potential, research in certain aspects of the MK and Q biosynthesis in some organisms has moved ahead of that of *E. coli*. The MK and the phylloquinone biosynthetic pathways (the reactions of biosynthesis are identical except the prenylation) are unique to bacteria and plants respectively and are absent in humans and animals. Hence, there is great commercial interest in discovering chemicals that will inhibit the enzymes of the pathway. Such chemicals will be of use as herbicides and chemotherapeutic agents against pathogens such as multi drug resistant *Mycobacterium tuberculosis* and Methicillin-resistant *Staphylococcus aureus* (MRSA). With the idea of designing drugs, the crystal structure of MenB from *M. tuberculosis* has been solved by two different groups (70, 161) and that of *S. aureus* was recently reported (163). Hence in this review whenever there is work on organisms that is not available in *E. coli* it will be included. It is expected that the crystal structures of enzymes will be similar if not identical in most organisms since they perform chemically identical reactions.

Most of the information concerning the biosynthesis of MK and Q was obtained with *E. coli* by using isotopic tracers, by the isolation of mutants, and accumulation of intermediates and enzyme assays. Due to space limitations, only a general account is given here; for more information, several comprehensive reviews should be consulted (7, 8, 10, 12, 13, 53, 54, 163, 168). Both MK and Q are derived from the shikimate pathway and as such have some common structural features. The quinone nucleus of Q is derived directly from chorismate while that of MK is from isochorismate via chorismate. The prenyl side chain on the nucleus of both is derived from prenyl PPi and the methyl groups are derived from S-adenosylmethionine. In addition, MK biosynthesis requires 2-ketoglutarate and thiamine PPi, coenzyme A and ATP as cofactors. The biosynthesis of Q under aerobic conditions has the additional requirements for oxygen, flavoprotein, and NADH. Finally, it should be noted that the Q biosynthetic pathway in prokaryotes differs in several respects from that of eukaryotes (71, 103, 107, 122, 152).

In spite of the fact that both quinones originate from the shikimate pathway, there are several important differences.

1. In the formation of the quinoid nuclei, the pathway for Q diverges at chorismate with the loss of a pyruvoyl group, due to the action of chorismate lyase, resulting in the formation of a benzenoid aromatic acid which is used as the framework on which the rest of the molecule is constructed. MK biosynthesis diverges at isochorismate by the addition of succinic semialdehyde-TPP anion derived from 2-ketoglutarate resulting in the formation of 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid (SEPHCHC). In the subsequent reaction, the pyruvoyl group is eliminated resulting in the prearomatic compound 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC). This is then aromatized to a benzenoid aromatic acid and used as the framework for the construction of the rest of the molecule as shown in Fig. 2.
2. In Q biosynthesis, the prenyl side chain is introduced at an early stage (second step) with the retention of the aromatic carboxyl group. Conversely, while in MK biosynthesis, prenylation is the next to last step and is accompanied by a decarboxylation.

3. In MK biosynthesis all the enzymes in the pathway up to the prenylation are soluble (next to the last step). In Q biosynthesis the enzymes are membrane bound except for the first enzyme.

4. In MK biosynthesis, methylation of the carbon of the nucleus is the last step while the terminal step of Q biosynthesis is the methylation of a hydroxyl group. In addition, in Q biosynthesis a second O-methylation and C-methylation take place in the middle portion of the pathway. Surprisingly, both the C-methylation involved in MK biosynthesis and the C-methylation in Q biosynthesis are carried out by the same C-methyltransferase.

5. Q biosynthesis under aerobic conditions requires the introduction of OH groups by reactions involving oxygen; anaerobic Q and MK biosynthesis utilize oxygen atoms derived from water.

MK BIOSYNTHESIS

The MK biosynthetic pathway has been elucidated on the basis of tracer experiments, isolation of mutants blocked in the various steps, isolation and identification of intermediates accumulated by the mutants and by enzyme assays. Early isotopic tracer experiments with various bacteria established that methionine and prenyl PPi contribute to the methyl and prenyl substituents of the naphthoquinone. The early isotopic tracer studies and other work have been reviewed by Bentley and Meganathan (12). In 1964, Cox and Gibson observed that [G-^{14}C] shikimate was incorporated into both menaquinone and ubiquinone by *E. coli*, thus providing the first evidence for the involvement of the shikimate pathway (25). Chemical degradation of the labelled isolated menaquinone (MK-8) showed that essentially all of the radioactivity was retained in the phthalic anhydride. It was concluded that "the benzene ring of the naphthoquinone (sic) portion of vitamin-K_2 arises from shikimate in *E. coli*"; (25). The authors further suggested that shikimate was first converted to chorismate before incorporation into MK. A more complete chemical degradation of the menaquinone derived from labelled shikimate established that all seven carbon atoms were incorporated (18). The remaining three carbon atoms of the naphthoquinone ring were shown to be derived from the middle three carbons of 2-ketoglutarate with the loss of both carboxyl groups (17, 132, 133).

These studies established the immediate precursors of menaquinone as shikimate and the noncarboxyl carbon atoms of 2-ketoglutarate forming the naphthoquinone nucleus. The methyl and isoprenoid side chains were also shown to be derived from S-adenosylmethionine and an isoprenyl alcohol pyrophosphate ester, respectively. Subsequently it was shown that the benzenoid aromatic compound *o*-succinylbenzoate (OSB) (29) and the naphthalenoid aromatic compound 1,4-dihydroxy-2-naphthoate (DHNA) (12, 134) were incorporated into the naphthoquinone ring of menaquinone. This work was confirmed by the demonstration that *menB* and *menA* mutants of *E. coli* excrete OSB and DHNA, respectively, into the culture medium (173). During a study of the biosynthesis of OSB by growing cultures of *E. coli menB* it was demonstrated that carbon atom one of the glutamate (2-ketoglutarate) was lost and consequently not incorporated into OSB (108). The isotopic labeling pattern is summarized in Fig. 3.

Formation of isochorismate (Compound IV -------> V)

The first synthesis of OSB **(IX)**, from chorismate **(IV)** and 2-ketoglutarate in the presence of TPP by cell-free extracts of *E. coli* was obtained by Meganathan (105) (Fig. 4). However, it had been suggested that isochorismate **(V)** was a much more attractive precursor than chorismate on chemical grounds (29, 53). Evidence in support of this hypothesis was provided (11, 36, 167).

Isochorismate is a common intermediate in the biosynthesis of the siderophore enterobactin and MK. The conversion of chorismate to isochorismate in enterobactin biosynthesis is mediated by the enzyme isochorismate synthase encoded by the *entC* gene (119, 162). The dual role of isochorismate led to the question as to whether the *entC* encoded isochorismate synthase (EntC) was supplying the isochorismate required for both pathways. Kaiser and Leistner (72) reported the isolation of a Tn10 insertion in the *entC* gene that had lost simultaneously the ability to form enterobactin and MK. It is generally accepted that the *entC* gene is derepressed under iron deficiency and repressed under iron sufficiency (47, 116). Enterobactin is required only under aerobic conditions due to the poor solubility and the consequent unavailability of iron in the Fe^{3+} form. When *E. coli* is grown anaerobically, iron is present in the highly soluble Fe^{2+} form. Hence, the synthesis of enterobactin is unnecessary for the acquisition of iron by the cell under anaerobic conditions (47, 116).

In contrast, MK is required under anaerobic conditions (12). Further, when the organism is grown with fumarate, trimethylamine-*N*-oxide (TMAO) or dimethylsulfoxide (DMSO) as electron acceptor, the presence of MK is obligatory (12, 48, 106, 112). When oxygen or nitrate are the electron acceptors, the aerobic quinone, ubiquinone is used by *E. coli* (84). Thus, while the conditions that favor the biosynthesis and function of Q are compatible with the biosynthesis of enterobactin, they are incompatible with the biosynthesis of MK.

These apparent contradictions raised some intriguing questions. How does *E. coli*, growing aerobically under iron deficiency when *entC* is fully derepressed, prevent the synthesis of MK? Further, under anaerobic conditions, how does *E. coli* prevent the synthesis of enterobactin when MK synthesis is induced? This paradox might be resolved if the *entC* gene is regulated by iron in the presence of oxygen and by MK requirement in the absence of oxygen. To study the regulation of the *entC* gene, an *entC-lacZ* operon fusion was constructed and the expression of β-galactosidase monitored under various conditions. It was found that the β-galactosidase was fully derepressed at low concentration of iron and repressed at high iron concentrations under both aerobic and anaerobic growth conditions (91, 93).

These results raised the question as to how *E. coli* is able to synthesize MK anaerobically when growing in the presence of high concentrations of iron? How does the organism prevent the excess production of MK under iron deficient aerobic conditions when *entC* is fully derepressed? To answer these questions, anaerobic growth of an *entC*::*Tn5* mutant was tested on glycerol medium with TMAO, DMSO or fumarate as electron acceptors. The mutant was able to grow at the same rate as the parent, even in the presence of high concentrations of iron. Further, the mutant produced as much MK as the parent (91, 93, 104). These results provided clear evidence for the presence of an alternate isochorismate

synthase specifically involved in MK biosynthesis. As a first step in locating and identifying the gene encoding this alternate isochorismate synthase involved in MK biosynthesis, further sequencing upstream of the 5′ region of the *menD* gene was carried out. An open reading frame encoding a 430 amino acid protein exhibiting about 20% amino acid identity with EntC was identified as MenF (30, 31, 91).

The isochorismate synthase (MenF) encoded by the *menF* gene has been overexpressed and purified to homogeneity. The purified enzyme had a relative M_r of 48, 000 (30, 31) as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The native M_r, as determined by gel filtration chromatography, was 98, 000, thus establishing that the native enzyme is a homodimer (30). The enzyme showed a requirement for Mg^{2+} for maximal activity.

It is expected that the origin of the hydroxyl groups and the mechanism of the reaction of MenF and EntC will be identical. Four different mechanisms were proposed for the EntC enzyme (165). The origin of the hydroxyl could be from three possible sources: 1). molecular oxygen, 2). intramolecular transfer of the hydroxyl, or 3). from the solvent H_2O. While the incorporation of molecular oxygen is possible only in the case of aerobic organisms intramolecular transfer or the incorporation of hydroxyl from water can be carried out by both aerobes and anaerobes. Due to the reported absolute requirement of enterobactin for the chelation of iron during aerobic growth under iron deficiency (47, 116) one would expect the incorporation of molecular oxygen into the hydroxyl group. However, the absence of redox cofactor rules out the involvement of oxygen and evidence has been obtained demonstrating the incorporation of the C-6 hydroxyl from the solvent H_2O for EntC (165). Consistent with this result is the demonstration of anaerobic biosynthesis of enterobactin in *E. coli* (91).

Recently, the 3D structure of MenF has been determined and the catalytic mechanism probed by site directed mutagenesis and biochemical studies (80). Lys 190 has been identified as the active site base that assists in the attack by water at the C_2 carbon. An S_N2" reaction results in the rearrangement of 1–2, 5–6 double bonds resulting in the elimination of the C4 hydroxyl group (80). These findings are in complete agreement with a common mechanism proposed for the three chorismate-utilizing enzymes, anthranilate synthase (AS), 4-amino-4-deoxychorismate synthase (ADCS) and isochorismate synthase (IS) by He *et al* (55).

Formation of succinic semialdehyde-TPP (SS-TPP) anion and Michael addition to isochorismate (compound VI+V------>VIII)

During the studies on the biosynthesis of *o*-succinylbenzoate (OSB) **(IX)**, cell extracts of two groups of mutants designated as *menC* and *menD* blocked in the formation of OSB and requiring OSB for anaerobic growth on glycerol-fumarate medium were examined. Cell extracts of either mutant alone did not form OSB from chorismate **(IV)** and 2-ketoglutarate in the presence of thiamin pyrophosphate. However, extracts from both mutants in combination produced OSB, and extracts of *menC* mutants accumulated an intermediate, which was converted to OSB by extracts of *menD* mutants (110). The intermediate was found to be unstable, and on mild acid treatment yielded OSB and succinylbenzene. On the

basis of these properties and nuclear magnetic resonance data, the intermediate was identified as 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) **(VIII)** (36).

It has been postulated that the 2-ketoglutarate undergoes a TPP-dependent decarboxylation, with the formation of succinic semialdehyde anion of TPP **(VI)** (17, 105) and a requirement for TPP in the reaction was shown (110). The mechanism of decarboxylation is identical to that catalyzed by the first enzyme of the 2-ketoglutarate dehydrogenase complex (KGDH complex) (Fig. 5)(11, 99). Using a *sucA* mutant, (which lacks the first enzyme of the KGDH complex), and by selective removal of the KGDH complex it was established that the 2-ketoglutarate decarboxylase (KDC) involved in OSB synthesis is a separate enzyme (13, 99, 167).

Subsequent studies established that the succinic semialdehyde anion **(VI)** of TPP reacted with isochorismate **(V)** resulting in the formation of SHCHC **(VIII)** (11, 36, 167) as had been postulated previously (29, 53). A mechanism for this reaction has been proposed (Fig. 5) (10, 11, 103). When the complete nucleotide sequence of the *menD* gene was determined it was discovered that both SHCHC synthase and KDC activities are encoded by a single gene (120). This conclusion was further strengthened by overexpression and purification of the MenD protein and by showing that both activities co-purified during various steps of the purification process (14, 89).

However, recently, Guo and colleagues have shown that the formation of SHCHC from isochorismate and 2-ketoglutarate is a two step process requiring two different enzymes. The first enzyme MenD decarboxylates the 2-ketoglutarate and adds the resulting succinic semialdehyde anion of TPP **(VI)** to isochorismate **(V)** resulting in the formation of 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC) (**VII**) (67). The mechanism of formation of succinic semialdehyde anion of TPP and its addition to isochorismate is shown in Fig. 5. The stereochemistry of SEPHCHC was determined and shown to be (1R, 2S, 5S, 6S)-2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1--carboxylic acid (68). On the basis of these results, MenD was designated as SEPHCHC synthase (67).

SEPHCHC is an unstable compound which, in mildly basic solutions, spontaneously undergoes a 2, 5 elimination reaction resulting in the formation SHCHC and pyruvate. Crystallization and a preliminary X-ray analysis of MenD has been reported (148)

The *in vivo* conversion of SEPHCHC to SHCHC (compound **VII** to **VIII**) is carried out by (1R, 6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase encoded by the *menH* gene (Fig. 4 and 5). Surprisingly, MenH contains a Ser-His-Asp catalytic triad, which is typical of many proteases. This triad plays a critical role in enzyme activity since replacing any one of the three amino acids by alanine results in a dramatic decrease in catalytic activity (69). The structure of MenH from the enteric pathogen *Vibrio cholerae* has been determined (Pdb# IR3D).

The aromatization of SHCHC----->OSB (Compound VIII-------->IX)

Enzymatic removal of the elements of water from SHCHC **(VIII)** leads to the formation of the benzenoid aromatic compound OSB **(IX)** (Fig. 4). The first evidence for the presence of such an enzyme was obtained by the demonstration that cell free extracts of a *menD* mutant converted SHCHC (designated as "X" at the time) to OSB (110). This enzyme was subsequently designated as OSB synthase (126, 141). The gene encoding OSB synthase was cloned and its complete nucleotide sequence reported (141).

The enzyme was overexpressed, purified to homogeneity and its properties investigated. The enzyme required a divalent metal ion for activity like the other members of the enolase superfamily. The enzyme was shown to carry out the dehydration of SHCHC to OSB very efficiently with K_{cat} of ($19\pm1 s^{-1}$) and a K_{cat}/K_m of ($1.6 \pm0.3\times10^6 M^{-1}s^{-1}$) (121). OSB synthase was classified as a member of the enolase superfamily. Members of this superfamily carry out reactions initiated by abstraction of the α-proton from a carboxylate anion substrate to generate a stabilized enolate anion intermediate (3). As pointed out above, the reaction catalyzed by OSB synthase is a dehydration. It was proposed that the α-proton of the carboxylate substrate (SHCHC) is likely abstracted by a basic catalyst (one lysine) followed by the elimination of the β-hydroxyl group presumably by the assistance of an acid catalyst (a second lysine) (121).

The structure of OSB synthase from *E. coli* in complex with Mg^{2+} and *o*-succinylbenzoate was determined. It was found that OSB synthase is the only monomeric member of the enolase superfamily. The product OSB was found to be sandwiched between Lys 133 and Lys 235 located at the ends of the second and sixth β-strands. In addition, one carboxylate oxygen of the substrate is coordinated to the Mg^{2+} (159). Subsequently, the structure of OSB synthase from an inactive K133R mutant in complex with the substrate SHCHC was determined. It was found that Lys 133 is the single base/acid catalyst for the dehydration with the transient Mg^{2+} coordinated enolate anion intermediate. The dehydration was shown to follow a *syn*-stereochemical course (74). The mechanism and specificity of various members of the enolase superfamily including OSB synthase have been reviewed and should be consulted for further details of the reaction mechanism (42).

Cyclization of OSB to DHNA (Compound IX ---------> XII)

The conversion of the benzenoid aromatic compound OSB **(IX)** to the naphthalenoid aromatic compound 1,4-dihydroxy-2-naphthoate (DHNA) **(XII)**, was demonstrated by Bryant and Bentley (16). The process showed an absolute requirement for ATP and CoA. Hence, OSB-CoA **(X)** was suggested as an intermediate. Using extracts of *Mycobacterium phlei*, evidence was obtained for the presence of two enzymatic activities (OSB-CoA synthetase and DHNA synthase). The OSB-CoA was found to be an unstable intermediate, which spontaneously hydrolyzed to the spirodilactone form of OSB **(**Fig. 6, compound **XIX)**. Further, it was shown that during the formation of OSB-CoA, ATP was hydrolyzed to AMP and PP_i, which is typical of ligases forming CoA esters (109).

The CoA moiety was suggested to be on the aromatic carboxyl group (16, 109) and evidence in support of this suggestion was obtained (56, 57). However, in subsequent publications, it was reported that the CoA is located on the aliphatic carboxyl group (81, 82).

A group of *E. coli* mutants responding to DHNA **(XII)**, but not to OSB **(IX)** for anaerobic growth on glycerol-fumarate medium was analyzed for their ability to convert OSB to DHNA (143). None of the mutant extracts formed DHNA. However, when the cell extracts from different mutants were mixed with each other, one of the mutant extracts complemented with extracts of each one of the other three mutants and formed DHNA. To identify the nature of the enzymatic defect in these mutants, cell extracts from each one of these mutants were complemented with OSB-CoA synthetase and DHNA synthase from *M. phlei* described above, and assayed for DHNA formation. The single mutant whose extract was complemented by OSB-CoA synthetase, and therefore, lacking this enzyme was designated as *menE*. The other three mutants, whose extracts were complemented by DHNA synthase were designated as *menB* (143).

The *menE* gene was cloned and sequenced (140). The gene was overexpressed and the enzyme purified to homogeneity. The purified enzyme had subunits of M_r 49,000 and a native M_r of 185,000. Thus, the native enzyme appears to be a homotetramer. The K_m values for OSB, ATP and CoA were 16, 73.5, and 360 μM, respectively (88). By chemical inactivation and site directed mutagenesis studies, an essential histidine residue (His341) located in the ATP binding region has been identified as necessary for catalytic activity of the enzyme (15). Sequence analysis combined with the fact that OSB-CoA synthetase hydrolyses ATP to AMP and PP_i and requires CoASH for the reaction earns it membership in the acyl-adenylate/thioester forming superfamily of enzymes (19, 20). A mechanism for the reaction has been proposed (Fig. 6).

The *menB* gene was cloned and its complete nucleotide sequence determined (142). When the gene was overexpressed and the protein purified to homogeneity, the subunits were found to have a M_r of 32,000, while the native protein had a M_r 112,000 as determined by gel filtration. Thus, the enzyme is a homotetramer (103).

As discussed above, the substrate for MenB, OSB-CoA is highly unstable. Hence, for the assay of MenB, the required OSB-CoA is generated *in vitro* by coupling the reaction with the MenE reaction (12, 109). However, for the coupled assays, crude cell-free extracts were always used. Surprisingly, when the overexpressed and purified MenE and MenB enzymes were used in the coupled assay, DHNA formation was not observed. In order to determine the reasons for the lack of formation of DHNA, small amounts of a crude cell-free extract of *E. coli* were added to the reaction mixture, and this resulted in the restoration of activity in the incubation mixture. Hence it appeared that either a cofactor or another protein might be involved in the reaction.

On the basis of alignment and analysis of the sequence MenB was included in the enoyl-CoA hydratase/isomerase (crotonase) superfamily (172). The failure of the purified MenB to form DHNA in the complementation assay discussed above and its membership in the enoyl-CoA hydratase superfamily (where other members form CoA esters) it was suggested

that the product of MenB is DHNA-CoA **(XI)** rather than DHNA **(XII)** (103). Evidence in support of this prediction has been obtained in *Mycobacterium tuberculosis* where the product of MenB was identified by mass spectrometry as DHNA-CoA **(XI)** (161). The crystal structure of MenB as the native enzyme and in complex with acetoacetyl-CoA and DHNA-CoA respectively has been reported by Truglio *et al*. (161) and Johnston *et al*. (70). The highly conserved active site of MenB contained a deep pocket lined with Asp-192, Tyr-287 and hydrophobic amino acids. Site directed mutagenesis studies have established that Asp-192 and Tyr-287 are essential for enzymatic catalysis. On the basis of structural and mutagenesis studies, the authors have proposed a possible mechanism for cyclization of OSB-CoA to DHNA-CoA (161).

On the basis of amino acid sequence homology to thioesterases, an unidentified *orf*152 (*yfbB*) was postulated to carry out the conversion of DHNA-CoA → DHNA and designated as *menH* (103, 121). Evidence in support of the proposal was provided by experimental demonstration of thioesterase activity of the protein (86).

However, as discussed above (section ___), the MenH protein has been unequivocally demonstrated to carry out the conversion of newly discovered intermediate SEPHCHC to SHCHC and has been christened as SHCHC synthase (69). Thus, the enzyme responsible for the conversion of DHNA-CoA to DHNA remains to be identified.

Prenylation of DHNA to DMK (Compound XII-------->III)

The conversion of DHNA **(XII)** to DMK **(III)** in extracts of *E. coli*, was shown by Bentley (8). Shineberg and Young (146) were able to isolate a membrane-bound 1,4-dihydroxy-2-octaprenyltransferase. The *menA* gene encoding the enzyme has been cloned (155). The enzyme (MenA) has many features in common with 4-hydroxybenzoate octaprenyltransferase (UbiA) involved in the biosynthesis of ubiquinone. The two enzymes share a common pool of membrane bound octaprenyl diphosphate (146). The conversion of DHNA to DMK requires replacement of the carboxyl with the isoprenoid side chain. Prenylation and decarboxylation may occur in a single active site, since symmetry experiments exclude 1,4-naphthoquinone as an intermediate (4). Moreover, there has been no evidence for two separate reaction steps or enzymes. A carbocation mechanism based on the dimethylallyl tryptophan synthase reaction (40) has been proposed for the reaction (103). In addition, a quinol to quinone oxidation is required in which demethylmenaquinol is a likely intermediate; the oxidation to DMK is thought to be spontaneous.

Methylation of DMK to MK (Compound III--------->II)

DMK **(III)** is methylated to MK **(II)** by a methyltransferase, which uses *S*-adenosylmethionine as the methyl donor. In experiments with whole cells it was shown that all three hydrogen atoms of the methyl group of methionine are transferred to DMK (66). The conversion of DMK-3 to MK-3 was demonstrated in cell extracts using *S*-[$^{14}CH_3$]-adenosyl-L-methionine by Bryant and Bentley(16). An *ubiA* mutant of *E. coli* was found to accumulate DMK but not MK. This mutant is believed to be defective in methylation of DMK to MK, suggesting that this is a double mutant (35). In a subsequent study, it was shown that an *ubiE* mutant, blocked in the methylation of the ubiquinone biosynthetic

intermediate, 2-octaprenyl-6-methoxy-1,4-benzoquinol (OMB) **(XXV)** to 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol (OMMB) **(XXVI)** (174) accumulated DMK but not MK (171). Consistent with this observation is the simultaneous loss of *C*-methyltranferase activity towards both OMB and DMK and its restoration by a plasmid containing the *ubiE* gene (95, 171).

Q BIOSYNTHESIS

The ubiquinone biosynthetic pathway was elucidated largely due to the work of Gibson, Cox, Young and colleagues (45, 46). In 1964, it was observed by Cox and Gibson that [G-^{14}C]-shikimate was incorporated into ubiquinone, thus establishing that the quinone was derived from the shikimate pathway (25). Gibson and colleagues reasoned that since ubiquinone is required for aerobic electron transport, mutants deficient in its biosynthesis would grow fermentatively on glucose, but not aerobically on oxidizable substrates such as malate or succinate, as the sole source of carbon and energy. Mutagenized cultures were screened for the desired phenotype and potential mutants were analyzed for the presence or absence of ubiquinone (45). Using this procedure, a number of mutants were isolated and it was found that these mutants accumulated sufficient amounts of intermediates so that their structure could be determined by mass spectrometry and magnetic resonance spectrometry (45, 154).

The biosynthesis of the quinonoid ring and the various ring modification reactions in *E. coli* have been reviewed (71, 73, 104, 152). A mechanistic perspective on the various reactions has been provided (6, 103). As pointed out in the introduction, in *E. coli* and *Salmonella*, the first committed step in the biosynthesis of Q is the formation of 4-hydroxybenzoate from chorismate by the cytoplasmic enzyme chorismate lyase. The 4-hydroxybenzoate formed is attached to the membrane bound octaprenyl diphosphate by a membrane bound octaprenyltransferase. For the subsequent reactions, all the substrates and enzymes are in a membrane bound complex. In this chapter, the intermediates, genes and enzymes involved in the various reactions are presented first. This will be followed by a brief description of the membrane bound multienzyme complex and the reported interactions of certain enzymes in the complex with each other.

Conversion of chorismate to 4-hydroxybenzoate (Compound IV--------->XX)

The elimination of pyruvate from chorismate **(IV)**, results in the formation of 4-hydroxybenzoate (4-HB) **(XX)** (Fig. 7). This aromatizing reaction is the first committed step in the biosynthesis of Q and is catalyzed by the enzyme, chorismate lyase, encoded by the *ubiC* gene (94). The *ubiC* gene has been cloned; the enzyme was overexpressed and purified to homogeneity. The UbiC is a monomer of 165 amino acids from which the N-terminal methionine is post-translationally removed resulting in the functional enzyme. The enzyme has a molecular weight of 18.645 Da and functions as a monomer. The K_m was reported to be around 6–10 μM (117, 147). The purified enzyme failed to accept isochorismate as a substrate, but did convert 4-amino-4-deoxychorismate to 4-aminobenzoate (117). Thus, it appears that the enzyme is unable to distinguish between the hydroxyl group and the amino group at the C-4 position. Walsh *et al*. (165) have proposed a 1,2-elimination of the elements of pyruvate for the aromatization similar to that of anthranilate synthase reaction.

The C-4 H of chorismate is abstracted by the enzyme and loss of the C-3-enolpyruvyl group then results in the formation of the 4-hydroxybenzoate (4-HB). It has been reported that the enzyme was inhibited by 4-HB but not pyruvate (147). In a subsequent study, Holden et al. circumvented the rapid influence of product inhibition on the initial reaction rate by using progress curve analysis of stopped-flow kinetic measurements. Under these conditions the K_m increased by about 3-fold to 29 μM (60). The enzyme releases the pyruvate quickly and retains the 4-HB with a 10-fold higher affinity (Kp = 2.1 μM) (39).

The crystal structure of UbiC protein has been solved. The wild type enzyme tended to aggregate and precipitate even in the presence of reducing agents and salt. To circumvent this problem, two surface-accessible cysteines at sequence positions 14 and 81 were converted by site directed mutagenesis into serine. This mutant enzyme C14S/C81S, designated as CCSS, showed greatly improved solubility and stability with minimal effect on the catalytic properties (60). The crystal structure of the enzyme from the double mutant at 1.4-Å and the wild-type enzyme at 2.0-Å in complex with the product, 4-HB was determined. The core of the chorismate lyase consisted of 6-stranded antiparallel β-sheet without spanning helices and novel connectivity. The product, 4-HB was shown to be bound in an internal cavity behind two flaps which completely covers and shields the product from the solvent. Three hydrogen bonds link the product to the internally charged side chains of Arg76 and Glu155 and two additional hydrogen bonds link it to the flap atoms 34 N and 114N. These five hydrogen bonds play a direct role in binding the product. There are three additional hydrogen bonds that link the flaps together and further enhance product retention (150).

To further clarify and understand the unusual ligand binding and the mechanism of reaction additional structures of mutant enzymes, enzyme inhibitor complexes and mutant enzyme inhibitor complexes were studied. When a high resolution crystal structure (1.0 Å) of the enzyme substrate complex was examined, a substrate sized internal cavity was found behind flaps near the product binding site. The crystal structure (2.4 Å) of the enzyme complexed with the inhibitor vanillate showed that the flaps were partly opened when compared to the product bound enzyme.

An active site mutant enzyme G90A complexed with the product was examined at a resolution of 2.0 Å. It was found that the presence of the additional methyl group in the mutant enzyme resulted in the enlargement of the 4-HB binding pocket by about 1 Å. However, all the 8-hydrogen bonds involved in product binding in the wild-type enzyme are maintained. When the kinetic properties of the mutant enzyme were compared with the wild-type enzyme it was found that the product inhibition increased by about 40%. The wild-type enzyme had a Kp-value of 1.5 (±0.2) μM versus 0.9 (±0.1) μM for the mutant. The increase in product inhibition in the mutant is attributed to the presence of the additional methyl group acquired in the conversion of glycine to alanine. The K_m values did not change while the K_{cat} value of the mutant decreased to 0.9 (±04.)S^{-1} from 1.4 (±0.2)S^{-1}.

When the G90A mutant enzyme was bound with the inhibitor vanillate, the structure at 1.9 Å showed two vanillate molecules. One of the vanillate molecules occupied the product site normally occupied by 4-HB and the second molecule of vanillate occupied an adjacent site

or cavity. The two sites were found to be connected by a tunnel that is open partly on both ends. The product binding site was designated as the primary ligand site and the adjacent site (additional site) where the second vanillate molecule binds was designated as the secondary ligand site (150). On the basis of structural studies summarized above in combination with molecular modeling, molecular dynamics and binding measurements with inhibitors, a model has been proposed to account for catalytic, product binding, and product release mechanisms.

It has been proposed that the enzyme operates by a two site or tunnel mechanism (150). According to this mechanism, the enzyme contains bound 4-HB in the primary site (designated as primary ligand site 1°). When the substrate binds to the second site (designated as secondary ligand site 2°), it promotes the release of the product from the primary site. As the product 4-HB is released from the primary site, the substrate chorismate moves to the primary site. In the primary site the substrate is unstable and it is rapidly converted to the products 4-HB and pyruvate. Since pyruvate is small, it exits rapidly from the primary site while the 4-HB is retained in the bound state and the process is repeated in a cyclic manner.

Prenylation of 4-hydroxybenzoate (Compound XX-------->XXI)

The prenylation of 4-hydroxybenzoate **(XX)** to 3-octaprenyl-4-hydroxybenzoate **(XXI)** is carried out by the enzyme 4-hydroxybenzoate octaprenyltransferase encoded by the *ubiA* gene. The enzyme is membrane bound and requires octaprenyl diphosphate and Mg^{2+} (175). In addition to octaprenyl diphosphate, the enzyme could incorporate geranyl, farnesyl, phytyl, or solanesyl diphosphate as a side chain precursor (35, 111). This lack of specificity also extends to the aromatic substrate; thus, 4-aminobenzoate can replace 4-hydroxybenzoate as a substrate (35). Recently, it has been shown that the enzyme accepts a wide variety of benzoic acid derivatives as substrates. As already mentioned, replacing the C-4 hydroxyl with an amino group did not affect reactivity. However, replacing the hydroxyl with a methoxy group was not tolerated. Compounds substituted at C-5 with OH, NH_2, Cl, or CO-CH_3 groups were used as substrates by the enzyme. Similarly, compounds with hydroxyl groups at C-4, C-5 and C-6 or hydroxyl group at C-4, C-6 and methyl group at C-5 were substrates (169).

The prenyl transfer reactions are electrophilic substitution reactions. The reaction mechanism probably includes a carbocation (43); evidence for this proposal comes from studies on the related enzyme dimethylallyltryptophan synthase (40).

Formation of 2-octaprenylphenol (Compound XXI -------->XXII)

The conversion of 3-octaprenyl-4-hydroxybenzoate **(XXI)** to 2-octaprenylphenol **(XXII)** was demonstrated by Cox *et al*. (26). The enzyme responsible for this conversion was named 3-octaprenyl-4-hydroxybenzoate decarboxylase. The presence of decarboxylase was also observed by El Hachimi *et al*. (35). The enzyme activity was absent in *ubiD* mutants (26).

When cell extracts were prepared using a French press, centrifuged at 30,000×g, and the supernatant further centrifuged at 150,000×g for 3 hrs, most of the activity remained in the soluble fraction, establishing that the enzyme separated from the membrane. A 24-fold purified preparation of the enzyme was obtained. The molecular weight of the enzyme was reported to be Mr 340,000 (98). For optimal activity, the enzyme required Mn^{2+}, washed membranes or an extract of phospholipids, and an unidentified heat stable factor of molecular weight less than 10,000. The reaction was strongly stimulated by dithiothreitol and methanol. Since the substrate of the enzyme 3-octaprenyl-4-hydroxybenzoate is membrane bound and the enzyme is stimulated by phospholipid, it has been suggested that the enzyme normally functions in association with the cytoplasmic membrane *in vivo* (98). A reaction mechanism has been suggested (6, 103).

A number of *ubiD* mutants studied form about 20% of the wild type levels of Q, indicating that the mutants are leaky or there is an alternate enzyme for the reaction. However, the significance of any alternate carboxy-lyase in wild type strains has been questioned (98).

An alternate 3-octaprenyl-4-hydroxybenzoate decarboxylase encoded by the *ubiX* gene has been described in *S. typhimurium* which carries out the same reaction as the *ubiD* encoded enzyme (61). An *ubiX* gene showing 70% homology to the *S. typhimurium* gene has been identified in *E. coli* (118, 177). The *orf*′s encoding the two enzymes UbiD and UbiX have been identified from *E. coli* (178). Recently, a report has appeared suggesting that both UbiD and UbiX are required for the decarboxylation of 3-octaprenyl-4-hydroxybenzoate particularly during logarithmic phase of growth (49).

It has been reported that several *E. coli* strains including the enterohaemorrhagic O157:H7 contain in addition to UbiX a second paralog designated as Pad1. The amino acid sequence of this paralog was reported to have a 52% identity to UbiX and a slightly higher identity to *S. cerevisiae* phenylacrylic acid decarboxylase Pad1. The exact biochemical role of *E. coli* Pad remains to be determined (129).

Hydroxylation and methylation reactions

In the subsequent steps of the pathway, the 2-octaprenylphenol undergoes three hydroxylation reactions alternating with three methylation reactions resulting in the formation of ubiquinol **(XXVIII)** and then Q **(I)**. For convenience, the hydroxylation reactions are considered together and this will be followed by a description of the three methylation reactions.

1. Hydroxylation reactions—Three flavin linked monooxygenases are involved in the three hydroxylation reactions of the pathway with three hydroxyl groups being introduced at positions C-6, C-4 and C-5 of the benzene nucleus, respectively. The three reactions are:

1. 2-octaprenylphenol **(XXII)** → 2-octaprenyl-6-hydroxyphenol **(XXIII)**,
2. 2-octaprenyl-6-methoxyphenol **(XXIV)** → 2-octaprenyl-6-methoxy-1,4-benzoquinol **(XXV)**

3. 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol **(XXVI)** → 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol **(XXVII)**.

Mutants blocked in each of these hydroxylation reactions were isolated and designated as *ubiB*, *ubiH*, and *ubiF*, respectively.

Consistent with their metabolic block, *ubiB* mutants accumulate 2-octaprenylphenol **(XXII)** (26, 176). However, the predicted product of the UbiB reaction **(XXIII)** has never been isolated and characterized and it may not occur as a free intermediate (2, 176).

As part of the genome project, when the sequence was annotated, *ubiB* was considered identical to that of *fre* and *luxG* (28). In subsequent studies, an *orf* previously designated *yigR* was identified as *ubiB*. An insertion mutant was isolated and was shown to accumulate the expected intermediate in the pathway, 2-octaprenylphenol (compound **XXII**). As mentioned above, the expected product of the reaction 2-octaprenyl-6-hydroxyphenol (**XXIII**) could not be isolated (125).

Mutants blocked in the methylation of 2-octaprenyl-6-hydroxyphenol (**XXIII**) to 2-octaprenyl-6-methoxyphenol (**XXIV**) have been isolated (*ubiG*::kan) (see methylation reactions below). However, surprisingly, these mutants also failed to accumulate the expected intermediate before the block 2-octaprenyl-6-hydroxyphenol (**XXIII**) thus supporting the suggestion that it may not occur as a free intermediate (2, 176).

Mutants unable to convert **(XXIV)** to **(XXV)** have been isolated with the gene being designated as *ubiH* (176). The *ubiH* gene is identical to the *visB* gene and confers a photosensitive phenotype due to the accumulation of **(XXIV)** (115).

The final hydroxylation in Q biosynthesis is the conversion of **(XXVI)** to **(XXVII)** and mutants blocked in the reaction were isolated and characterized. As expected, these mutants, designated as *ubiF*, accumulated **(XXVI)** which was isolated and identified (174). The *ubiF* gene was identified as *orf*391 and the product accumulated by insertion mutants in this *orf* was found to be **(XXVI)** (92).

Under aerobic conditions, the origin of the oxygen atoms of Q was determined by ^{18}O labeling experiments. Cultures were grown on the oxidizable carbon source succinate, under strictly aerobic conditions in a defined atmosphere of $^{18}O_2$. The Q was isolated from these cultures and subjected to mass spectral analysis. The spectrum showed several prominent peaks with *m/z* values differing from that of normal Q by +6 establishing that ^{18}O had been incorporated. Further, it was demonstrated that the ^{18}O was incorporated at positions 4, 5, and 6 (2).

The nature of the hydroxylation reactions discussed above has been investigated. A *hemA* mutant defective in the biosynthesis of cytochromes was able to convert 2-octaprenyl-[^{14}C] phenol to ^{14}C-labeled Q-8, ruling out the involvement of the cytochrome P-450 monooxygenase system, and suggesting the involvement of flavin linked monooxygenases in these reactions (75). A mechanism analogous to that proposed for the flavin dependent tyrosine hydroxylase (166) has been suggested by Begley *et al*.(6).

When grown anaerobically, with glycerol as a carbon source and fumarate as an electron acceptor, *E. coli* forms considerable quantities of Q (50–70% of aerobically grown cells). Mutants blocked in the various non-hydroxylating reactions of the pathway such as *ubiA*, *ubiD* and *ubiE*, remain Q deficient under both aerobic and anaerobic conditions, establishing that the same genes and enzymes participate under both aerobic and anaerobic conditions (1).

In contrast, the three groups of mutants blocked in the three oxygenases discussed above, *ubiB*, *ubiH*, and *ubiF*, were able to synthesize Q under anaerobic conditions providing evidence that specific hydroxylases are involved in the anaerobic pathway (1). These hydroxylases likely derive the hydroxyl groups from the solvent H_2O similar to EntC and MenF reactions discussed above.

2. Methylation reactions—Two methylations on O and one on C involved in the pathway are:

1. 2-octaprenyl-6-hydroxyphenol **(XXIII)** → 2-octaprenyl-6-methoxyphenol **(XXIV)** (i. e. *O*-methylation).
2. 2-octaprenyl-6-methoxy-1,4-benzoquinol **(XXV)** → 2-octaprenyl-3-methyl-6 methoxy-1,4-benzoquinol **(XXVI)** (i. e. C-methylation).
3. 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol (OMHMB) **(XXVII)** → ubiquinol **(XXVIII)** (i. e. O-methylation).

The methylation steps alternate with the three hydroxylations described above introducing methyl groups at 6-OH, at the ring C-3 and the 5-OH group, respectively. The three methyl groups are derived from methionine (66), with S-adenosylmethionine being the actual methyl donor.

The C-methylase responsible for the methylation of ring C-3 is encoded by the *ubiE* gene. Mutants blocked in the methylation accumulate the substrate of the enzyme, 2-octaprenyl-6-methoxy-1,4-benzoquinol **(XXV)** (174). The UbiE enzyme is non-specific and carries out the methylation of the menaquinone intermediate, DMK **(III)** → MK **(II)** in addition to its role in the methylation of **(XXV)** → **(XXVI**) (95) (discussed in MK).

During the screening for mutants blocked in the *O*-methylation reactions, mutants blocked in the methylation of 6-OH were not obtained. However, mutants blocked in the methylation of the 5-OH were isolated, designated as *ubiG* and were shown to accumulate compound **(XXVII)** which was isolated and characterized (154). Further, the *ubiG* mutants being leaky formed about 10% of the wild type levels of Q (1). In subsequent studies, it was reported that the O-methylase encoded by the *ubiG* gene is non-specific and that it carries out the methylation of both 6-OH and 5-OH groups (62). This lack of specificity also extends to the presence of other groups on the benzoquinone ring; the enzyme in addition, methylates 3, 4-dihydroxy-5-hexaprenylbenzoquinol to 3-methoxy-4-hydroxy-5-hexaprenylbenzoquinol. The reported leakiness of the *ubiG* mutant, mentioned above, likely allowed sufficient intermediate **(XXIII)** to be methylated at the 6-OH resulting in the formation of **(XXIV)**, which was subsequently converted to **(XXVII)** and methylated at the 5-OH resulting in the

formation of **(XXVIII)** and Q. A *ubiG*::kan mutant has been isolated. However, surprisingly, this mutant failed to accumulate the expected intermediate before the block 2-octaprenyl-6-hydroxyphenol **(XXVIII)**. Two possible reasons have been advanced for the failure to detect compound **(XXVIII)**. Firstly, as mentioned above, compound **(XXVIII)** may not occur as a free intermediate (2, 176). Secondly, it has been suggested that the compound may be highly reactive due to the presence of the catechol moiety and hence degraded (62).

Organization of Q biosynthetic enzymes into a complex

As discussed above, not all the enzymes involved in Q biosynthesis have been studied in cell free extracts. Among the enzymes studied, chorismate pyruvate-lyase (UbiC) is a cytoplasmic enzyme, while 4-hydroxybenzoate octaprenyltransferase (UbiD) is firmly membrane bound. Two other enzymes that have been studied, 3-octaprenyl-4-hydroxybenzoate carboxy-lyase and 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol methyltransferase, are considered to be normally associated with the membrane (97, 98). The association of enzymes with membrane is supported by the isolation of a 2-octaprenyl [U-^{14}C] phenol **(XXII)** charged enzyme complex of molecular mass Mr of 2×10^6 containing at least 12 proteins ranging from 40,000 to 80,000 Mr from cells grown anaerobically on glycerol/fumarate medium in the presence of 4-hydroxy[U-^{14}C] benzoate. When this complex was incubated with S-adenosylmethionine, NADH, NADPH, Mg^{2+}, and a cytoplasmic enzyme of molecular weight of about 20,000 (probably a methyltransferase) (77) in the presence of oxygen, all of the ^{14}C-labelled phenol was converted to Q (76). This complex, therefore, contains the oxygen dependent Q-8 biosynthetic apparatus. In anaerobically grown cells, this apparatus which is charged with 2-octaprenylphenol may be kept in a standby position. When oxygen becomes available, Q-8 biosynthesis can be effectively turned on (76, 77). Since this complex was isolated without detergent treatment, it was thought that it had broken from the membrane as a distinct and native domain. This complex contains in addition to a high level of 2-octaprenylphenol and low levels of Q, phospholipid, and other membrane proteins (76, 77).

Based on studies with a thiol-sensitive mutant (IS16), it was reported that there is genetic evidence for interaction between UbiX and UbiG proteins (50). The IS16 mutant had point mutations resulting in change of a single amino acid in UbiX (S98R) and UbiG (L132Q) when compared to the sequence of the same two proteins in *E. coli* K_{12}. Complementation of this mutant with either *ubi*X from *E. coli* K_{12} strain (*ubiX* K_{12}) or *ubiG* K_{12} restored the wild-type phenotype. In conntrast, while an *ubi*G insertion mutant was rescued by complementation by *ubi*G K_{12} it was not rescued by *ubi*X K_{12} (50). Rescue of Q deficient phenotypes can be achieved by levels of Q that are significantly lower than that present in the wild-type strains (50). These studies were cited as providing supporting evidence for the polypeptide complex described by Knoll (77) discussed above.

Regulation of Q biosynthetic genes

It is known that the quinone composition of *E. coli* is influenced by the availability of oxygen. Cells grown under vigorous aeration contain 2 to 3-fold higher concentrations of Q compared to DMK and MK. Under anaerobic conditions, the MK and DMK concentration

increases 2 to 3-fold while the concentration of Q decreases (12, 152). The mechanism of this regulation is not completely understood. Shestopalov *et al*. (145) have shown that chloramphenicol had no effect on these changes, suggesting post-translational regulation of quinone levels. Further, these authors have shown that mutations in the regulatory systems of Fnr and Arc had no effect on the quinone pool. Suzuki *et al*. (156) studied the regulation of the *ubiA* gene using plasmid borne *lacZ* fusions and showed that the gene is catabolite repressed by glucose. A similar study on plasmid borne *ubiG* gene also showed glucose catabolite repression (44). SØballe and Poole (151) studied the transcriptional regulation of *ubiC-lacZ* in a monolysogen and showed that the expression was higher aerobically than anaerobically. It was further reported that glucose repressed expression while anaerobic growth in the presence of alternate electron acceptors, nitrate and fumarate did not affect expression. Further it was shown that *ubiC* was negatively regulated by transcriptional regulators Fnr and IHF (151).

In a recent study, the expression of the operon fusions *ubiC'*-lacZ$^+$, *ubiCA'*-lacZ$^+$, and *ubiA'*-lacZ$^+$ were studied. In glycerol media under aerobic conditions the highest level of expression was observed with the operon fusion *ubiC'*-lacZ$^+$. Compared with the *ubiC'*-lacZ$^+$, the *ubiCA'*-lacZ$^+$ operon fusion showed 26% of the activity while the *ubiA'*-lacZ$^+$ operon fusion had an activity of 1%. Thus, the *ubiC* gene is regulated by the upstream promoter while the *ubiA* gene lacks its own promoter (90). The effect of fermentable and oxidizable carbon sources on the expression of *ubiC'*-lacZ$^+$ was determined. The expression was low in the case of fermentable carbon source glucose; increasing glucose concentration resulted in increased repression. In the presence of oxidizable carbon sources the expression increased 2- to 3-fold. In both fermentable and oxidizable carbon sources, supplementation of the medium with casamino acids resulted in decrease in expression. Aerobically, deficiency in both Q and MK or MK alone resulted in a 2-fold increase in expression compared with wild-type cells. In the strain carrying the *arcA* mutation, under anaerobic conditions the expression was from 25% to 50% higher than the anaerobically grown wild-type strain, while in the *fnr* mutant the activities did not change (90). The lack of regulation by FNR is in agreement with the absence of binding site (139). In the case of the *narXL* mutant, the activity increased 50% anaerobically and 137% in the presence of NO_3^- In the presence of other electron acceptors, O_2, fumarate, and TMAO, the activities were from 70% to 90% higher than that of the wild-type (90).

The expressions of the two genes involved in the decarboxylation of 3-octaprenyl-4-hydroxybenzoate, *ubiD* and *ubiX* were studied using LacZ operon fusions. During aerobic growth the expression of both genes depended on the carbon source: succinate>glycerol>glucose. Mutations in *fnr*, *arcA* or *hemA* increased the expressions of both genes. During anaerobic growth in LB medium glucose strongly repressed the expression of *ubiD* but not *ubiX* (178).

Consequences of mutations in *ubi* genes

Certain pleotrophic properties which will be of value in isolating and/or characterizing mutants are described here. As described above, *ubi* mutants were isolated by their inability to utilize succinate or other reduced compounds as carbon sources.

Hypersensitivity to thiols such as dithiothreitol (DTT), 2-mercaptoethanol and 1-thioglycerol was demonstrated in *ubiX* and *ubiD* mutants (177). Subsequently, it was shown that a *ubiCA* insertion mutant also exhibited this property (153). Thiol sensitivity is likely a common property of all Q deficient strains since a respiratory chain is essential for the maintenance of the disulfide bond forming system (78, 79).

An *E. coli* mutant resistant to the antibiotic and antitumor agent phleomycin was isolated. The mutant was also found to be resistant to bleomycin and unable to grow on succinate as the sole source of carbon and resistant to the lethal effects of heating at 52°C. The *suc*$^{-}$ phenotype and mapping data led to the conclusion that the mutant was defective in the *ubiF* gene. To confirm the observed properties, known *ubiA*, *ubiD* and *ubiF* mutants were compared with the newly isolated mutants. It was found that they also exhibited these properties (24). Recently, an *ubiCA* mutant was shown to exhibit the pleotrophic phenotype, being resistant to heat, linolenic acid and phleomycin. In addition, it has been shown that Q is involved in superoxide scavenging, and in protection against oxidative stress mediated by $CuSO_4$ or H_2O_2 (153).

A mutant showing partial resistance to streptomycin was found to be defective in the *ubiF* gene. Membranes of this strain accumulated 2-octaprenyl-3-methyl-6-methoxy-1,4 benzoquinol **(XXVI)** but not Q. A previously characterized *ubiF* mutant was found to show reduced uptake of gentamycin. At present, there is no evidence implicating Q in aminoglycoside antibiotic uptake and these observations are attributed to the general impairment of respiratory capacity (114).

Mutations in the Q biosynthetic pathway (*ubiD, ubiB*, and *ubiG*) led to the lack of flagellar synthesis and motility (5, 58). An *ubiA men*$^{+}$ strain was motile anaerobically and nonmotile aerobically, while mutants blocked in Q and MK were found to be nonmobile under both aerobic and anaerobic conditions. Thus, it appears that a functional electron transport system is essential for motility and flagellar synthesis.

Mutants lacking Q, MK or both have been isolated and the role of quinones in electron transport to oxygen and nitrate has been studied (164).

Functions of isoprenoid quinones MK and Q

The roles of MK in the anaerobic respiratory chains and Q in the aerobic respiratory chains are well established. For details on the role of the quinones other reviews and chapters in this series should be consulted (32, 41, 51, 52, 64, 73, 123, 124, 131, 152). EcoSal Chapter The Aerobic and Anaerobic Respiratory Chain of Escherichia coli and Salmonella enterica: Enzymes and Energetics covers the aerobic and anaerobic metabolism and respiratory chains.

Biosynthesis of Isoprenoid side chain of MK and Q

E. coli and other Gram negative bacteria synthesize the isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by the mevalonate-independent pathway also known as non-mevalonate pathway (other names are deoxyxylulose phosphate or methylerythritol phosphate pathway) The IPP condenses with DMAPP and the prenyl chain is elongated to

the 40-carbon octaprenyl diphosphate (OPP). The details of side chain biosynthesis are beyond the scope of this chapter. A number of reviews are available on the topic (21, 34, 37, 38, 63, 87, 103, 107, 135–138, 149, 158).

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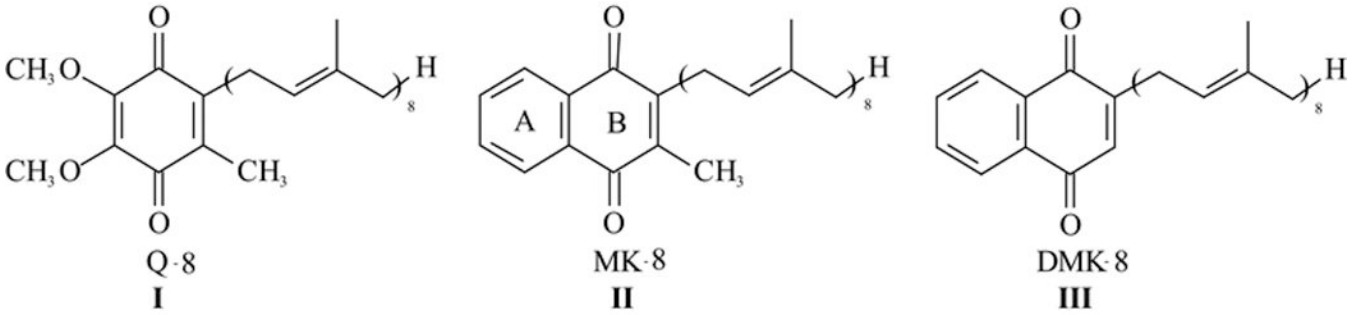

Fig. 1.

Structures of major quinones found in *E. coli*. In the structure of MK, the A ring and B ring of the naphthoquinone are shown.

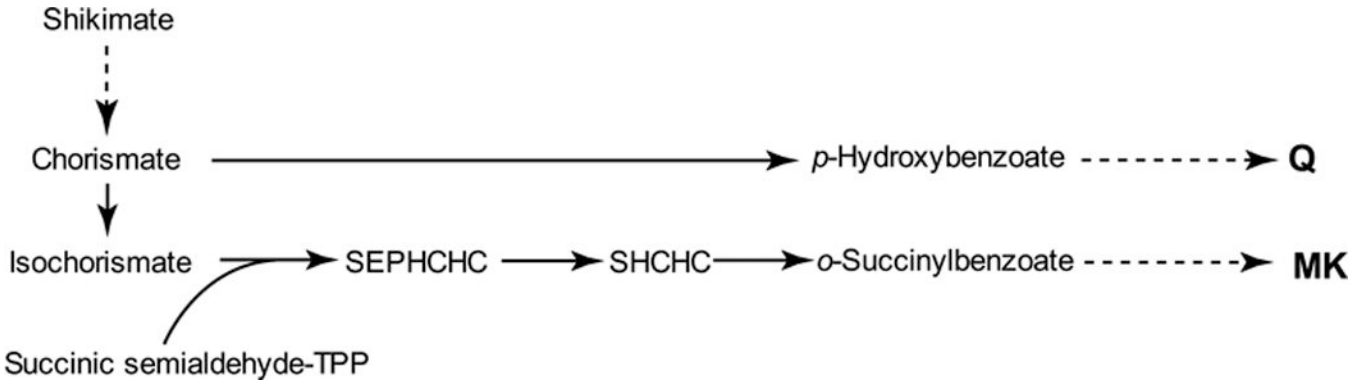

Fig. 2.
Formation of Q and MK.

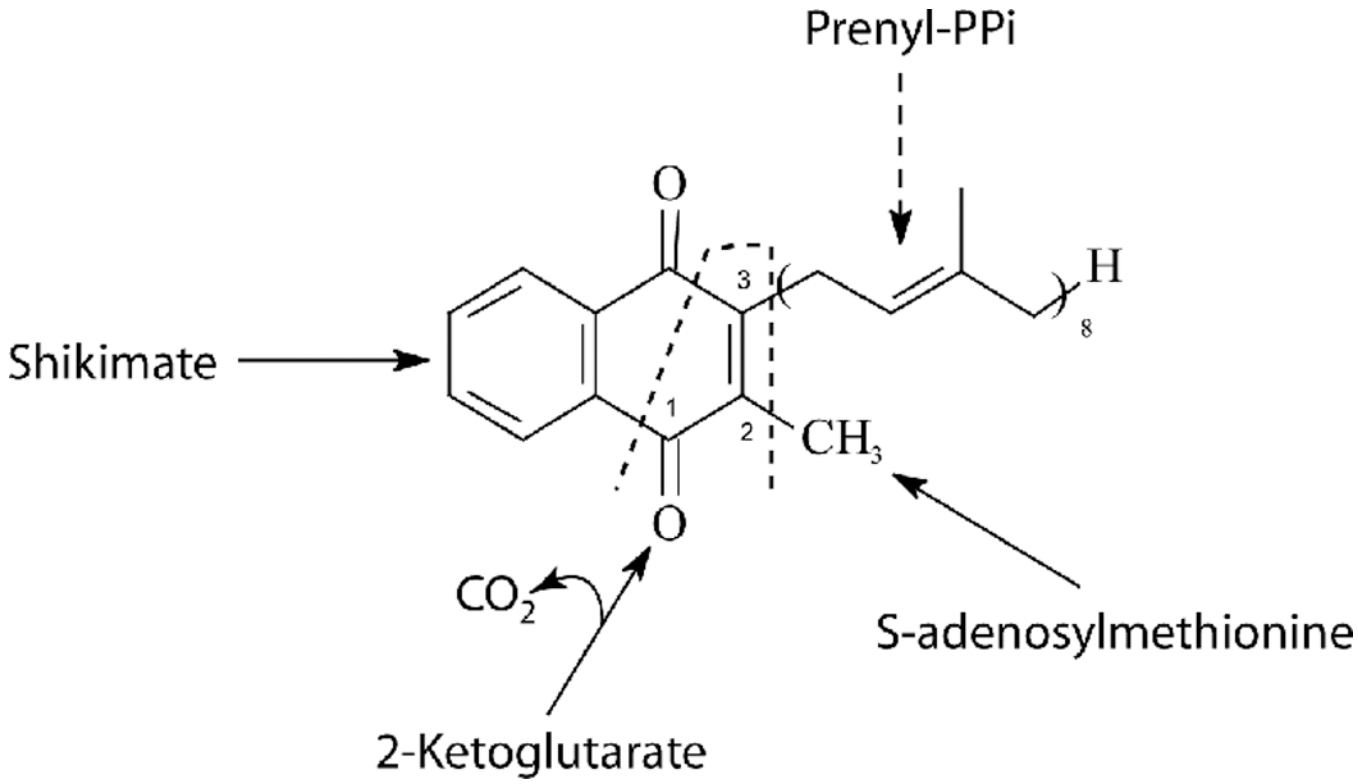

Fig. 3.
Primary biosynthetic precursors of menaquinones.

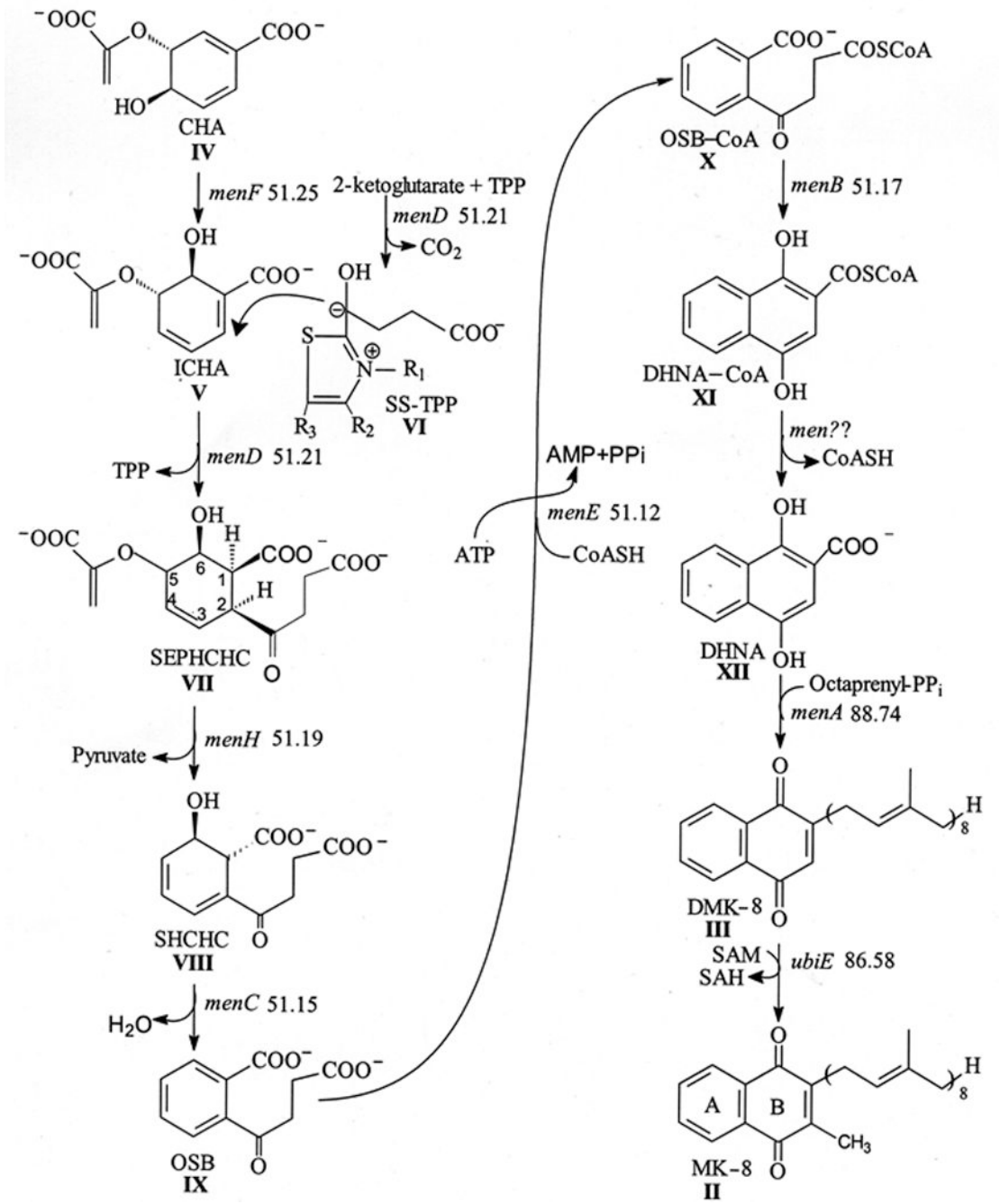

Fig. 4.

Menaquinone biosynthetic pathway. Each compound in the pathway is identified by its abbreviation and a Roman numeral. CHA, chorismate; ICHA, isochorismate; SS-TPP, succinic semialdehyde-TPP, R_1 = pyrimidine component of TPP, R_2 = CH3, R_3 = $CH_2CH_2OP_2O_6^{3-}$; SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; OSB, *o*-succinylbenzoate; OSB-CoA; *o*-succinylbenzoyl-CoA; DHNA-CoA, 1,4-dihydroxy-2-napththoyl-CoA; DHNA, 1,4-dihydroxy-2-napththoate; DMK-8, demethylmenaquinone

(may be initially formed as a quinol). MK-8, menaquinone; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. The genes encoding the enzymes are shown for each reaction followed by their location on the chromosome in min. The gene encoding the thioesterase for the conversion of DHNA-CoA (compound **XI**) to DHNA (compound **XII**) remains to be identified and is shown as men? ?.

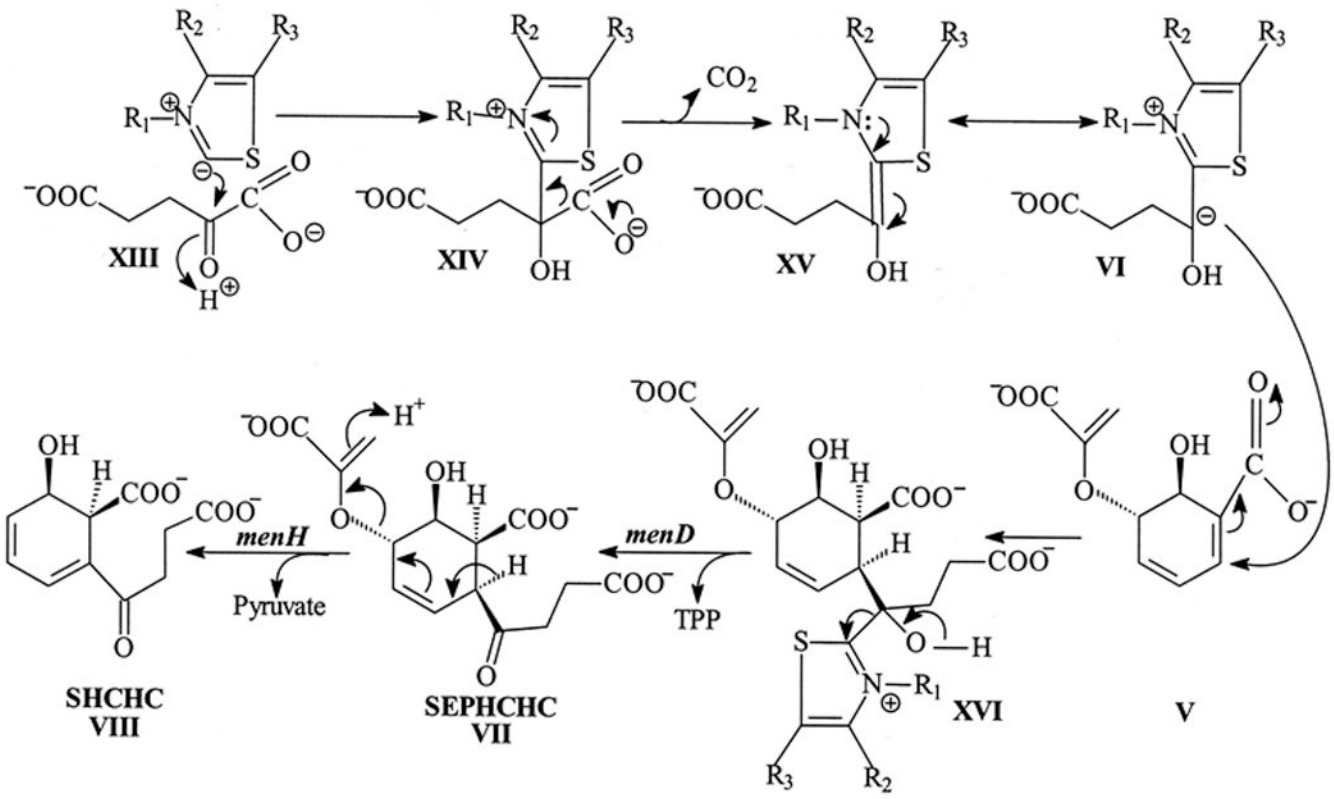

Fig. 5.

Proposed mechanism of succinic semialdehyde-TPP anion formation and SHCHC **(VIII)** synthesis. Only the thiazole ring of the TPP is shown since it is the active site of the molecule. For R1, R2, and R3 see the legend to Fig. 4. The reactions from **(XIII)** to SEPHCHC **(VII)** are carried out by MenD and the conversion of SEPHCHC **(VII)** -----> SHCHC **(VIII)** is by MenH.

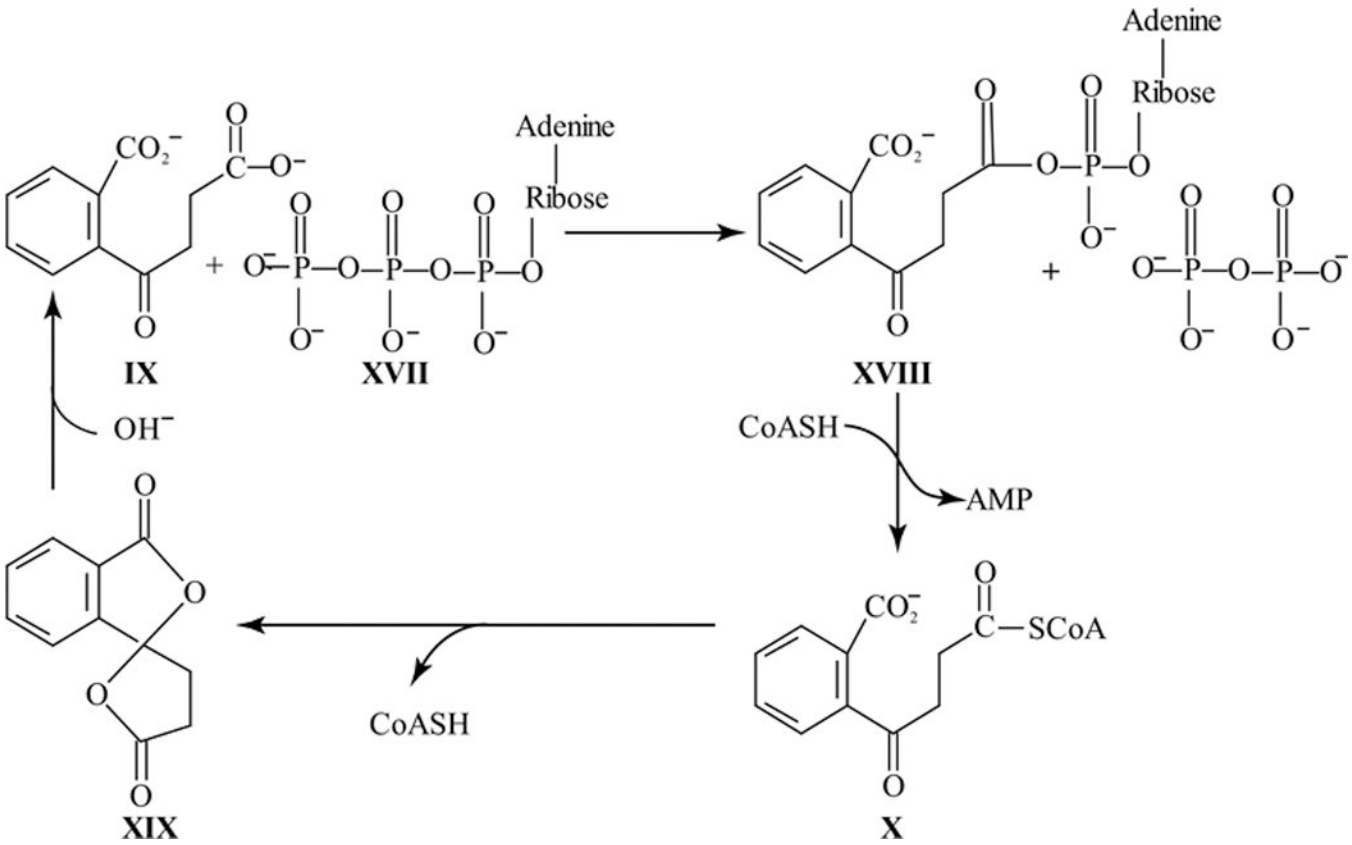

Fig. 6.

Proposed mechanism of formation of acyl adenylate of OSB **(XVIII)** and its subsequent conversion to OSB-CoA **(X)**. The conversion of OSB-CoA **(X)** to spirodilactone of OSB **(XIX)** is non-enzymatic.

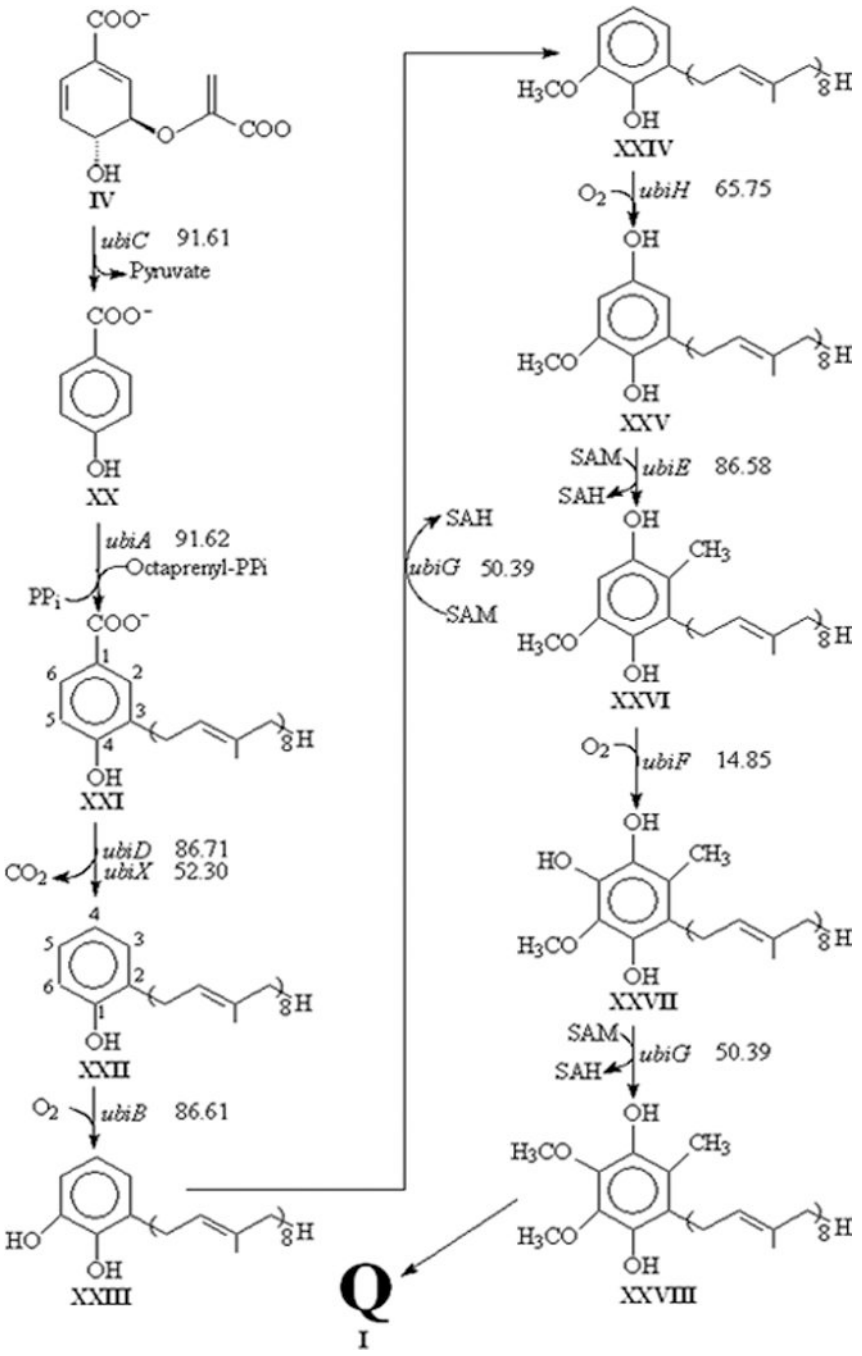

Fig. 7.

Ubiquinone biosynthetic pathway. Each compound in the pathway is identified by a Roman numeral. Under anaerobic conditions, there are alternate hydroxylases for the three enzymes incorporating molecular oxygen (UbiB, UbiH, and UbiF). It should be noted that in compound **(XXI)**, the chemical numbering system locates the prenyl side chain at the C-3 carbon; in compound **(XXII)** and subsequent intermediates, the prenyl side chain is assigned to C-2. Compounds **(XXV), (XXVI)**, and **(XXVII)**, are drawn in the quinol form. Some authors draw these structures in the quinone form. For other abbreviations see legend to Fig.

4. The chemical names for the intermediates of the pathway are as follows: **(IV)**, chorismate; **(XX)**, 4-hydroxybenzoate; **(XXI)**, 3-octaprenyl-4-hydroxybenzoate; **(XXII)**, 2-octaprenylphenol; **(XXIII)**, 2-octaprenyl-6-hydroxyphenol; **(XIV)**, 2-octaprenyl-6-methoxyphenol; **(XXV)**, 2-octaprenyl-6-methoxy-1,4-benzoquinol; **(XXVI)**, 2-octaprenyl-3-methyl-6 methoxy-1,4-benzoquinol; **(XXVII)**, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol; **(XXVIII)**, ubiquinol; **(I), Q**, ubiquinone. The conversion of **(XXVIII)** to **(I)** is thought to be non-enzymatic.