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Menaquinone (Vitamin K_2) Biosynthesis: Cloning, Nucleotide Sequence, and Expression of the *menC* Gene from *Escherichia coli*

V. SHARMA, R. MEGANATHAN,* AND M. E. S. HUDSPETH

Department of Biological Sciences and The Plant Molecular Biology Center
Northern Illinois University, DeKalb, Illinois 60115

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The benzenoid aromatic compound *o*-succinylbenzoic acid is formed by dehydration of the prearomatic compound 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid by the enzyme *o*-succinylbenzoate synthase, encoded by the *menC* gene. A 1.3-kb *Pst*I-*Pvu*II fragment was found to complement the *menC* mutation. The complete nucleotide sequence of this fragment revealed a single open reading frame of 954 bp capable of encoding a 35-kDa protein. A consensus sequence for a ribosomal binding site but no promoter consensus sequences were found. However, the first base of the initiating codon of this open reading frame overlaps the upstream *menB* gene termination codon, suggesting an operon-like organization for these genes. Consistent with this suggestion, the *menB* promoter can initiate transcription of the *menC* gene.

Menaquinone (MK), or 2-methyl-3-prenyl-1,4-naphthoquinone, plays an essential role in several anaerobic electron transport systems (9, 21). The complete metabolic pathway for the biosynthesis of MK has been reviewed (2, 3). The benzenoid compound *o*-succinylbenzoic acid [OSB; 4-(2′-carboxyphenyl)-4-oxobutyric acid] is the first aromatic intermediate of the pathway (6). Subsequently, cell extracts of *Escherichia coli* were shown to be capable of synthesizing OSB when incubated with chorismate and α-ketoglutarate (α-KG) in the presence of thiamine pyrophosphate (TPP) (11). Two groups of mutants blocked in the formation of OSB, designated as *menC* and *menD*, have been reported (8, 13). When incubated with chorismate, α-KG and TPP, cell extracts of *menC* mutants accumulated an intermediate which was converted to OSB by extracts of the *menD* mutant (13). On the basis of nuclear magnetic resonance data, this intermediate was identified as 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC) (7). It was further demonstrated that the immediate precursor of SHCHC is isochorismate (7, 23) rather than chorismate as previously postulated (6). The enzyme responsible for the conversion of SHCHC to OSB is encoded by the *menC* gene and has been designated OSB synthase (15, 16). The formation of SHCHC, its conversion to OSB, and the enzymes and genes involved are summarized in Fig. 1.

Of the five identified MK biosynthetic genes, four (*menB*, *menC*, *menD*, and *menE*) are clustered at 48.5 min on the *E. coli* chromosome (1, 20). These four genes have been cloned (20), and the complete nucleotide sequences of two, *menB* and *menD*, have been reported (14, 15, 19). In this paper, we report on the cloning, nucleotide sequence, and expression of the *menC* gene.

(A preliminary report of these findings has appeared elsewhere [18].)

Bacterial strains and growth conditions. The *E. coli* strains and plasmids used are listed in Table 1. The plasmids constructed in this study are described in Fig. 2. Cultures were routinely stored in glycerated L broth and grown on L agar. Recombinant clones containing the inserts in pUC18 and pUC19 vectors were selected on L agar plus 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 50 μg of ampicillin per ml. Plasmid complementation assays using *menC* mutants were performed on glycerol minimal medium containing 50 μg of ampicillin per ml, using trimethylamine *N*-oxide (TMAO) as the electron acceptor (19). Enzymatic complementation assays were performed with cell extracts from Trypticase soy broth-grown cultures harvested and prepared as previously described (14, 19).

DNA isolation and construction of plasmids. Plasmid DNAs were isolated by the alkaline lysis procedure of Birnboim and Doly (4) and purified in ethidium bromide-CsCl gradients. Plasmids used in this study consisted of fragments derived from the *men* cluster insert of pGS23 (20) and cloned into pUC18 and pUC19 as shown in Fig. 2. For some constructs, individual fragments were electroeluted from 0.8 or 1.0% agarose gel slices by using an IBI (New Haven, Conn.) unidirectional electroeluter. Ligation and transformation procedures were as described previously (10). The host strain for initial transformations was either *E. coli* HB101 or *E. coli* JM83.

DNA sequencing and sequence analysis. DNA sequences were determined by the dideoxynucleotide chain termination method (17), using the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{32}P]dATP (ICN, Costa Mesa, Calif.). The initial DNA sequence was determined from alkaline-denatured double-stranded plasmid DNAs. Compressed regions were resolved by single-stranded sequencing from equivalent M13 clones by using either dITP or 7-deaza-dGTP. Sequencing reactions were primed with universal, reverse, or sequence-generated synthetic oligonucleotide primers synthesized on an Applied Biosystems (Foster City, Calif.) 391 DNA synthesizer. All nucleotide positions were confirmed by sequencing of the complementary strands. Nucleic acid and deduced protein sequences were analyzed by using the Pustell DNA sequence analysis program (IBI) and the Genetics Computer Group program (University of Wisconsin Biotechnology Center, Madison, Wis.).

Cloning and sequencing of the *menC* gene. Cloning of the *E.*

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* Corresponding author.

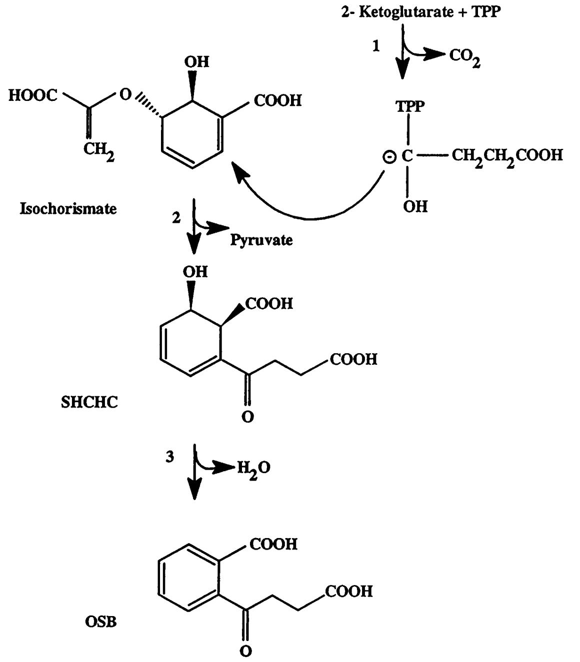

FIG. 1. Formation of SHCHC from isochorismate and α-KG and its subsequent conversion to OSB. 1, α-KG decarboxylase; 2, SHCHC synthase; 3, OSB synthase. Enzymes 1 and 2 are encoded by the *menD* gene, and enzyme 3 is encoded by the *menC* gene.

coli men gene cluster into pBR322 as pGS23 and identification of the complementing regions for the various *men* genes have been described elsewhere (20). In that report, a 3.3-kb *Pst*I fragment subcloned as pGS50 was shown to complement the *menC* mutants (20). To more precisely localize the *menC* coding region, the *Pst*I fragment and derivatives (Fig. 2) were cloned into pUC18 and transformed into either HB101 or JM83. These constructs were then assayed for the ability to restore anaerobic growth on glycerol-TMAO medium when transformed into a *menC* mutant. The smallest clone obtained, pMS29, contained a 1.3-kb *Pst*I-*Pvu*II insert (Table 2). The 5′ end of this insert overlaps by 250 bp the 3′ end of the insert in recombinant plasmid pMS1, which contains the *menB* coding region (19). The DNA sequence of this *Pst*I-*Pvu*II insert was determined to further characterize the *menC* locus. Contained within the insert (Fig. 3) is a single open reading frame (ORF) of 954 bp potentially encoding a 35-kDa protein. The first base of the putative initiating codon overlaps the upstream *menB* gene (19) termination codon. An acceptable ribosomal binding site, consisting of three of six consensus nucleotides (CGGAAT), is also located within the 3′ end of the *menB* sequence, but no obvious promoter consensus sequences are present. A search of the GenBank and SwissProt data bases failed to identify any significant amino acid homologies with the *menC* ORF.

TABLE 1. *E. coli* K-12 strains and plasmids used

Strain or plasmid	Relevant genotype	Reference(s) or source
E. coli strains		
PL2024	*gal trpA trpR iclR rpsL*	8, 13
JRG862	*menC1* ((OSB requiring mutant of PL2024)	8, 13
JLP200	JRG862(pJP101)	15
RM860	JRG862(pITS557)	This study
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Laboratory stock
JM83	F^- *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [φ80 Δ(*lacZ*)M15]	Laboratory stock
Plasmids		
pJP101	*menD*$^+$ Ap^r in pKK223-3	15
pITS557	*entC*$^+$ Ap^r in pGEM3Z	22
pUC18		IBI
pUC19		IBI

Verification of the identified *menC* ORF as the OSB synthase gene. The validity of the identified *menC* ORF as the OSB synthase-encoding locus was initially confirmed by in vitro enzymatic complementation assays and subsequently by the use of deletion plasmids. Cell extracts of the wild-type strain PL2024, the *menC* strain JRG862, and strain JRG862 complemented with pMS29 were assayed for OSB synthase activity as follows. Since SHCHC, the substrate for the reaction, and isochorismate, one of the immediate precursors of SHCHC, are unstable and commercially unavailable, they were generated in vitro from chorismate, α-KG, and TPP, using cell extracts from strains carrying plasmids expressing amplified levels of enzymatic activity. Strain RM860 [JRG862 *menC* (pITS557)] contains the *entC*$^+$ gene on the plasmid and produces amplified levels of isochorismate synthase, and strain JLP200 [JRG862 *menC* (pJP101)] contains the *menD*$^+$ gene on the plasmid and overproduces SHCHC synthase and α-KG decarboxylase. For the assay of OSB synthase activity, the procedure of Popp et al. (16) was used with modifications. The incubation mixture consisted of 35 μl of 1 M Tris-HCl buffer (pH 9.0), 25 μl of chorismate (5 mg/ml), 20 μl of α-KG (3.6 mg/ml), 20 μl of TPP (2.8 mg/ml), extracts from strains RM860 and JLP200 (1.5 to 2.0 mg of protein), and the extract from the strain to be assayed (0.15 mg of protein). The volume was adjusted to 300 μl with water and incubated at 37°C for 30 min. The OSB formed was determined by high-performance liquid chromatography. Authentic OSB was used as a standard. Protein was determined by the method of Bradford (5), using chemicals supplied by Bio-Rad Laboratories, Richmond, Calif.

As indicated in Table 2, the parent strain PL2024 produced 300 nmol of OSB/h/mg of protein. In contrast, the *menC* strain bearing pMS29 produced 660 nmol of OSB/h/mg of protein. This elevated level of OSB formation in the presence of pMS29 established the presence of the *menC* reading frame in the 1.3-kb *Pst*I-*Pvu*II insert.

The pMS29 deletion plasmids pMS32 and pMS32-1 (Fig. 2) were also used to confirm the legitimacy of the identified *menC* ORF. In the deletion analysis, each plasmid was transformed into the *menC* mutant and assayed for its ability to restore anaerobic growth on glycerol-TMAO medium. Neither a *Pst*I-*Bgl*II deletion encoding the first 44 N-terminal amino acid residues (pMS32) nor a *Bcl*I-*Pvu*II deletion encoding the last 60 C-terminal residues (pMS32-1) of the *menC* ORF restored anaerobic growth (Table 2). These results corroborate the location of the *menC* gene in the *Bgl*II-*Bcl*I region of the insert and thus the identified *menC* ORF.

Evidence for the expression of *menC* from the *menB* promoter. The poor complementation of the *menC* strain by the 3.3-kb *Pst*I-*Pst*I insert in the pBR322-derived plasmid pGS50 was attributed to the absence of promoter sequences (20). This is in contrast to the high level (two times that of the wild type) found by using the pUC18-derived pMS29 with the

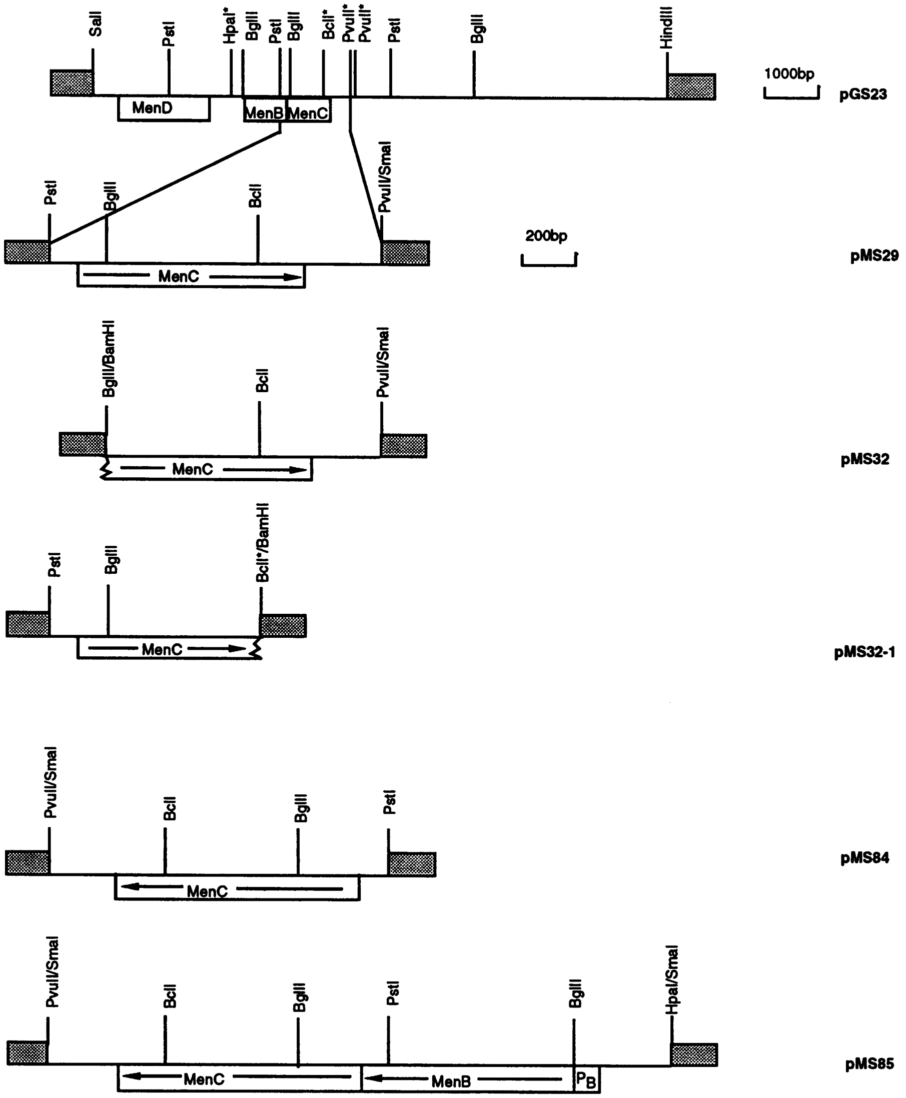

FIG. 2. Construction of *menC* recombinant plasmids. pMS29 is a pUC18 subclone of the 3.3-kb *Pst*I-*Pst*I insert of pGS23 (20); pMS32 and pMS32-1 are 5′ and 3′ deletions of pMS29, respectively; pMS84 is the reverse orientation of pMS29 in pUC19; pMS85 is a fusion of pMS48 (19), containing *menB* and a putative promoter (P_B), with pMS84 in pUC19. Stippled regions are vector sequences. Restriction enzymes with additional sites in pGS23 are marked with asterisks.

1.3-kb *Pst*I-*Pvu*II insert. However, similar results with use of a promoterless *menB* insert had demonstrated that elevated enzymatic activity resulted from the use of the pUC18 *lacZ′* promoter (19). Given the apparent absence of promoter sequences flanking the *menC* ORF and given its single-base overlap with the *menB* ORF, we made constructs to assay the effect of a plasmid containing genomic sequences extending from the *menB* promoter through *menC*. As the pMS29 insert is in the same orientation as the *lacZ′* promoter, a basal level of *menC* activity, independent of the *lacZ′* promoter, was initially established by reversing the orientation of the pMS29 insert as pMS84 and assayed for OSB production in the *menC* host. This activity was significantly less than that with use of pMS29. When the mutant carried pMS85, containing *menB* with its promoter plus *menC* (Fig. 2) in the same orientation as in pMS84, the level of OSB was the same as for the original pMS29-bearing strain.

TABLE 2. In vivo complementation and OSB formation in the presence of various *menC* plasmids[a]

Strain	Plasmid	In vivo complementation[b]	OSB formed (nmol/h/mg of protein)[c]
PL2024		+	300
JRG862		−	ND
JRG862	pMS29	+	660
	pMS84	(+)	116
	pMS32	−	ND
	pMS32-1	−	ND
	pMS85	+	652

[a] The plasmid-containing strain for enzymatic assays was the *menC* mutant JRG862.

[b] Assayed by anaerobic growth on glycerol-TMAO medium. +, growth; −, no growth; (+), poor growth.

[c] Activity has been corrected for the endogenous activity obtained, by incubating extracts of JRG862, JLP200, and RM860 under the assay conditions. ND, not detectable.

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                    M  R  S  A  Q  V  Y  R  W  Q  I  P  M  D  A  G  V  V  L  R  D  R  R  L  K  T  R  D  G
1201  AACGGAATCCGTAATGCGTAGCGCGCAGGTATACCGCTGGCAGATCCCCATGGACGCGGGGGTGGTTCTGCGCGACAGGCGGTTAAAAACCCGCGACGGG
         R  N  P  *

       L  Y  V  C  L  R  E  G  E  R  E  G  W  G  E  I  S  P  L  P  G  F  S  Q  E  T  W  E  E  A  Q  S  V  L
1301  CTGTATGTTTGCCTGCGTGAAGGCGAGCGCGAAGGGTGGGGGGAGATCTCCCCACTGCCGGGCTTCAGTCAGGAAACCTGGGAAGAGGCGCAAAGTGTGC

         L  A  W  V  N  N  W  L  A  G  D  C  E  L  P  Q  M  P  S  V  A  F  G  V  S  C  A  L  A  E  L  T  D
1401  TGCTTGCCTGGGTAAATAACTGGCTGGCAGGCGATTGCGAGCTACCGCAGATGCCTTCCGTGGCCTTTGGCGTAAGCTGTGCATTGGCAGAACTGACAGA

        T  L  P  Q  A  A  N  Y  R  A  A  P  L  C  N  G  D  P  D  D  L  I  L  K  L  A  D  M  P  G  E  K  V
1501  TACGTTGCCGCAAGCAGCCAACTACCGTGCGGCACCGCTGTGTAATGGCGATCCGGACGATCTGATCCTCAAACTTGCAGATATGCCAGGCGAGAAAGTG

       A  K  V  K  V  G  L  Y  E  A  V  R  D  G  M  V  V  N  L  L  L  E  A  I  P  D  L  H  L  R  L  D  A  N
1601  GCGAAGGTCAAAGTGGGATTGTACGAAGCGGTGCGCGACGGCATGGTGGTGAATCTGTTGCTGGAGGCAATTCCGGATCTGCATTTGCGTCTTGACGCAA

         R  A  W  T  P  L  K  G  Q  Q  F  A  V  N  P  D  Y  R  D  R  I  A  F  L  E  E  P  C  K  T  R  D  D
1701  ATCGCGCCTGGACACCGCTGAAAGGTCAGCAGTTTGCCGTTAACCCGGATTATCGCGACCGCATCGCGTTTCTCGAAGAGCCGTGCAAAACCCGCGATGA

        S  R  A  F  A  R  E  T  G  I  A  I  A  W  D  E  S  L  R  E  P  D  F  A  F  V  A  E  E  G  V  R  A
1801  TTCGCGAGCGTTTGCCCGTGAAACCGGCATTGCCATTGCCTGGGATGAAAGCCTGCGCGAGCCGGATTTTGCCTTTGTGGCTGAAGAGGGCGTGCGCGCG

       V  V  I  K  P  T  L  T  G  S  L  E  K  V  R  E  Q  V  Q  A  A  H  A  L  G  L  T  A  V  I  S  S  S  I
1901  GTAGTTATCAAACCCACGCTCACGGGCAGTCTGGAAAAAGTACGCGAGCAGGTACAGGCGGCGCACGCGCTGGGGCTGACGGCGGTGATCAGTTCTTCCA

         E  S  S  L  G  L  T  Q  L  A  R  I  A  A  W  L  T  P  D  T  I  P  G  L  D  T  L  D  L  M  Q  A  Q
2001  TTGAATCGAGCTTAGGCTTAACGCAACTGGCGCGGATTGCCGCCTGGTTAACGCCGGACACCATTCCAGGGCTGGACACGCTGGATCTGATGCAGGCGCA

        Q  V  R  R  W  P  G  S  T  L  P  V  V  E  V  D  A  L  E  R  L  L  *
2101  GCAGGTACGTCGCTGGCCGGGTAGCACGCTGCCTGTCGTGGAAGTTGATGCACTGGAGCGGTTGTTATGA
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FIG. 3. Nucleotide and deduced amino acid sequences of the *E. coli menC* gene. The noncoding strand is shown and numbered according to the upstream *menB* gene (19). Single-letter amino acid codes are indicated above the codons for *menC* and below for the 3′ region of *menB*. Asterisks represent termination codons. The putative ribosomal binding site is underlined.

Conclusions and discussion. SHCHC is the first committed intermediate in the MK biosynthetic pathway. It is formed from isochorismate and α-KG in the presence of TPP by the enzymatic activities SHCHC synthase and α-KG decarboxylase (15). Both of these activities are encoded by the *menD* gene (14). The enzyme OSB synthase, encoded by *menC*, converts the SHCHC to OSB (15, 16). Hence, extracts from *menC* mutants, when incubated with isochorismate, α-KG, and TPP, accumulate SHCHC (15, 16). We have cloned and identified an ORF in a *menC*-complementing fragment which, by enzymatic complementation and deletion analysis, has been shown to encode OSB synthase. This *menC* ORF contains 318 codons capable of generating a 35-kDa protein. This ORF appears to overlap the upstream *menB* gene by a single nucleotide, and it has an appropriate ribosomal binding site but no obvious promoter sequences.

The level of expression of the pMS29-bearing *menC* mutant was significantly higher than that reported for a similar region cloned into pBR322 in which the low level of expression was attributed to the absence of promoter sequences (20). As the insert in plasmid pMS29 is in the same orientation as the *lacZ′* promoter, we attribute this increase in *menC* expression to the upstream vector promoter, not to insert promoter sequences. This conclusion is supported both by the decreased level of expression with the insert in reverse orientation (pMS84) and by similar findings for *menB* (19). The inclusion of *menB* sequences with its promoter in the insert (pMS85) in the same orientation as in pMS84 dramatically elevated *menC* expression and suggested that *menC* can utilize the *menB* promoter. Nevertheless, the level of OSB synthase activity in these constructs remains less than expected for a multicopy plasmid. This latter observation, however, is consistent with similar reports for other MK biosynthetic genes like *menD* and *menB* (14, 15, 19). Even the placement of *menD* under the control of the *tac* promoter failed to generate the expected level of expression (15). The reason for the low level of expression of *men* genes remains obscure.

Nucleotide sequence accession number. The 970-bp sequence data reported in this paper appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number L07256.

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