THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 19, Issue of May 9, pp. 12380–12383, 1997

*Printed in U.S.A.*

# Identification of a Novel Gene Cluster Participating in Menaquinone (Vitamin $K_2$) Biosynthesis

## CLONING AND SEQUENCE DETERMINATION OF THE 2-HEPTAPRENYL-1,4-NAPHTHOQUINONE METHYLTRANSFERASE GENE OF *BACILLUS STEAROTHERMOPHILUS**

(Received for publication, February 10, 1997)

**Ayumi Koike-Takeshita‡, Tanetoshi Koyama§¶, and Kyozo Ogura¶‖**

*From the ‡Bio Research Laboratory, Toyota Motor Corporation, Toyota-cho 1, Toyota, Aichi 471-71, the §Department of Biochemistry and Engineering, Faculty of Engineering, Tohoku University, Aoba-ku, Sendai 980-77, and the ‖Institute for Chemical Reaction Science, Tohoku University, Aoba-ku, Sendai, Miyagi 980-77, Japan*

**We recently described the isolation and sequence analysis of a DNA region containing the genes of *Bacillus stearothermophilus* heptaprenyl diphosphate synthase, which catalyzes the synthesis of the prenyl side chain of menaquinone-7 of this bacterium. Sequence analyses revealed the presence of three open reading frames (ORFs), designated as ORF-1, ORF-2, and ORF-3, and the structural genes of the heptaprenyl diphosphate synthase were proved to consist of ORF-1 (*heps-1*) and ORF-3 (*heps-2*) (Koike-Takeshita, A., Koyama, T., Obata, S., and Ogura, K. (1995) *J. Biol. Chem.* 270, 18396–18400). The predicted amino acid sequence of ORF-2 (234 amino acids) contains a methyltransferase consensus sequence and shows a 22% identity with UbiG of *Escherichia coli*, which catalyzes *S*-adenosyl-L-methionine-dependent methylation of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone. These pieces of information led us to identify the ORF-2 gene product. The cell-free homogenate of the transformant of *E. coli* with an expression vector of ORF-2 catalyzed the incorporation of *S*-adenosyl-L-methionine into menaquinone-8, indicating that ORF-2 encodes 2-heptaprenyl-1,4-naphthoquinone methyltransferase, which participates in the terminal step of the menaquinone biosynthesis. Thus it is concluded that the ORF-1, ORF-2, and ORF-3 genes, designated *heps-1*, *menG*, and *heps-2*, respectively, form another cluster involved in menaquinone biosynthesis in addition to the cluster of *menB*, *menC*, *menD*, and *menE* already identified in the *Bacillus subtilis* and *E. coli* chromosomes.**

Prenyltransferases catalyze the fundamental isoprenoid chain elongation to produce prenyl diphosphates with various chain lengths and stereochemistries, which are led to such diverse isoprenoid compounds as steroids, carotenoids, glycosyl carrier lipids, prenyl quinones, and prenyl proteins.

Heptaprenyl diphosphate (HepPP)[1] synthase is one of the three prenyl diphosphate synthases in *Bacillus stearothermophilus*. It catalyzes the condensations of four molecules of isopentenyl diphosphate (IPP) with farnesyl diphosphate (FPP) to give HepPP, the precursor of the menaquinone (MK) side chain in this bacterium.

MK, or 2-methyl-3-prenyl-1,4-naphthoquinone, is a lipophilic nonprotein component of the electron transport chain in bacteria. In addition, MK is necessary for successful endospore formation and is involved in regulation of cytochrome formation in *Bacillus subtilis* (1). MK is synthesized in bacteria from isochorismate through a series of MK-specific reactions (Fig. 1). Five MK biosynthetic genes, *menA*, *menB*, *menC*, *menD*, and *menE*, have been identified, and four of them are shown to be clustered on the chromosomes of *Escherichia coli* (2–5) and *B. subtilis* (6, 7), although *menC* has not yet been identified in *B. subtilis* (8).

Previously, we reported the cloning of the genes that encode the HepPP synthase of *B. stearothermophilus* (9), which is composed of two nonidentical protein components. Sequence analyses of the gene regions revealed the presence of three open reading frames designated as ORF-1, ORF-2, and ORF-3. After several deletion experiments, we concluded that the structural genes of the HepPP synthase were ORF-1 and ORF-3, designated *heps-1* and *heps-2*, respectively. Then the next question is what is the function of ORF-2, which is located between the two structural genes of HepPP synthase.

A data base search for proteins similar to the predicted product of ORF-2 (234 amino acids) indicated that the ORF-2 product contains the methyltransferase consensus sequence and shows a 69% identity with GerC2, whose gene is one of the genes in the *gerC* cluster of *B. subtilis*, which is involved in spore germination (10), a 22% identity with UbiG of *E. coli*, which catalyzes *S*-adenosyl-L-methionine-dependent methylation of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone (11), and a 37% identity with the O251 of *E. coli*, whose function is not yet known (12).

To identify the function of ORF-2 as the 2-heptaprenyl-1,4-naphthoquinone methyltransferase gene, we constructed an expression system of ORF-2 and examined the enzymatic activity for catalysis of the terminal step in MK biosynthesis. This is the first report on the cloning of the gene encoding the methyltransferase catalyzing the terminal step in MK biosynthesis as well as on the existence of another gene cluster involved in MK biosynthesis.

* This work was supported by Grants-in-aid for Scientific Research 06240102 and 07680620 from the Ministry of Education, Science, and Culture, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D87054.*

¶ To whom correspondence should be addressed. K. Ogura: Tel.: 81-22-217-5621; Fax: 81-22-217-5620; E-mail: ogura@icrs.tohoku.ac.jp. T. Koyama: Tel.: 81-22-217-7271; Fax: 81-22-217-7293; E-mail: koyama@icrs.tohoku.ac.jp.

[1] The abbreviations used are: HepPP, heptaprenyl diphosphate; MK, menaquinone; IPP, isopentenyl diphosphate; FPP, (*E*,*E*)-farnesyl diphosphate; PCR, polymerase chain reaction; ORF, open reading frame; SAM, *S*-adenosyl-L-methionine; $^{14}$C-SAM, *S*-adenosyl-L-[methyl-$^{14}$C]methionine.

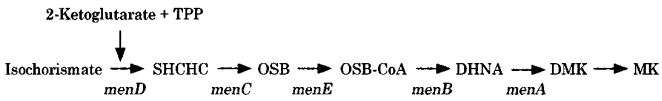


FIG. 1. **Biosynthetic pathway of menaquinone.** The abbreviations used are as follows: *TPP*, thiamine pyrophosphate; *SHCHC*, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; *OSB*, *o*-succinyl benzoate; *OSB-CoA*, *o*-succinyl benzoate-coenzyme A; *DHNA*, 1,4-dihydroxy-2-naphthoic acid; *DMK*, demethyl-MK. *Italics* show the genetic loci in mutant strains of each separate reaction step.

```
O251     1:-----------------------------------------------------------M-  1
COQ3     1:MGFIMLLRSRFLKVIHVRKQLSACSRFAIQTQTRCKSTDASEDEVKHFQELAPTWWDTDG 60

ORF2     1:MRQS-------------KEER----VHRVFENISAHYDRMNSVISFRRH-LKWRKDVMRR 42
GerC2    1:MQDS-------------KEQR----VHGVFEKIYKNYDQMNSVISFQQH-KKWRDKTMRI 42
O251     2:VDKSQETTHF-GFQTVAKEQKA-DMVAHVFHSVASKYDVMNDLMSFGIH-RLWKRFTIDC 58
UbiG     1:----------MNAEKSPVNHNVDHEEIAKFEAVASRWWDLEGEFKPLHRINPLRLGYIAE 50
COQ3    61:SQRILHKMNLTRLDFVQRTVRNQVKIQNPEIFVPGFNYKEFLPEYVCDNIQREMQESIET 120
DauC     1:---------------------MQDSSYKKQVTQAFDQSSSTYDRLGVEFFTPMGRRLVDI 39
PmtA     1:--------------------------MELDAVSRSYKRWAIYD-FSFGKVSESPRRRTAA 33

ORF2    43:MNV-QKGKKALDVCCGTADWT-IALAEAVGPEGKVYGLDFSENMLKVGEQKVKARG-LHNV 100
GerC2   43:MNV-KEGAKALDVCCGTADWT-IALAKAAGKSGEIKGLDFSENMLSVGEQKVKDGG-FSQI 100
O251    59:SGV-RRGQTVLDLAGGTGDLT-AKFSRLVGETGKVVLADINESMLKMGREKLRNIGVIGNV 117
UbiG    51:RAGGLFGKKVLDVGCGGGILA-ESMAREG---ATVTGLDMGFEPLQVAKLHALESGIQVDY 107
COQ3   121:NLDKRPEVSVLDVGCGGGILS-ESLARLKWV-KNVQGIDLTRDCIMVAKEHAKKDPMLEG- 178
DauC    40:SEPVTGER-VLDIGCGRGACLFPAAEKVGSQ-GCVHGIDIAPGMIEEARKEATERGLRNI- 97
PmtA    34:HVNARGGR-VLEVGVGTGLSLPLYSHR-----VAVTGIDFSHEMLARAREKVEEMGLEPVK 88
                  ####################
                      RegionI

ORF2   101:KLIHGNAMQLPFPDNSFDYVTIGFGLRNVPDYMTVLKEM-HRVTKPGGITVCLETSQPTL 159
GerC2  101:ELLHGNAMELPFDDDTFDYVTIGFGLRNVPDYLTVLKEM-RRVVKPGGQVVCLETSQPEM 159
O251   118:EYVQAXXEALPFPDNTFDCITISFGLRNVTDKDKALRSM-YRVLKPGGRLLVLEFSKPII 176
UbiG   108:VQETVEEHAAKH-AGQYDVVTCMEMLEHVPDPQSVVRAC-AQLVKPGGDVFFSTLNRNGK 165
COQ3   179:KI-NYECKALEDVTGQFDIITCMEMLEHVDMPSEILRHCWSRLNPEKGILFLSTINRDLI 237
DauC    98:SLMVMDAETPGFPARSFDLVMGSYSVIFLPDAVGALAR-YADILDHGGRIAFTSPV-FRA 155
PmtA    89:ELRQMDARELDFPDETFDTVVAMFLVSVVPEPERVVSE-MARVCRKGGEVVIVNH--FAR 145
                    ############                    ############
                     RegionII                        RegionIII

ORF2   160:FGFRQLYYFYFRFIMPLFGKL-LAKSYEEYSWL-QESAREFPGRDELAEMFRAAGFVDVE 217
GerC2  160:FGFRQAYFMYFKYIMPFFGKL-FAKSYKEYSWL-QESARDFPGMKELAGLFEEAGLKNVK 217
O251   177:EPLSKAYDAYSFHVXPRIGSL-VANDADSYRYL-AESIRMHPDQDTLKAMMQDAGFESVD 234
UbiG   166:SWLMAVVGAEYILRMVPKGTHDVKKFIKPAE-LLGWV-D-Q--TS--LKERHITG-LHY- 216
COQ3   238:SWFTTIFMGENVLKIVPKGTHHLSKYINSKE-ILAWFND-NY-SGQ-FRLLDLKG-TMY- 291
DauC   156:GTFPFLPPEFTPLIPQALLEHLPEQWRPE-ALVR-RFNSWLERAEDLVRTLEGCGYARLR 213
PmtA   146:DKGPLAAVE-KAL-AR-FENTL-G-WHSDFAIERVTADPRLEVVEQTPMSPGG-LFTFLR 199

ORF2   218:VKPYTFGVAAMHLGYKR---------------------------------------------- 234
GerC2  218:YHSFTGGVAATHIGWK---------------------------------------------- 233
O251   235:YYNLTAGVVALHRGYKF---------------------------------------------- 251
UbiG   217:-NPITNTFKLGPGVDVNYMLHTQN-K-------------------------------------- 240
COQ3   292:-LPYQGWVEHDCSDVGNYFMAIQRLN------------------------------------- 316
DauC   214:QSTSRCG---------------------------------------------------------- 220
PmtA   200:-FQRR---------------------------------------------------------- 203
```

FIG. 2. **Comparison of amino acid sequence of ORF-2 and other methyltransferases and related proteins.** The amino acids marked "#" represent the methyltransferase consensus sequences designated Regions I–III proposed by Ingrosso *et al.* (16). ORF2, this work. *GerC2*, product encoded by one of the genes in *B. subtilis gerC* cluster involved in spore germination (10); *O251*, unidentified *E. coli* protein (12); *UbiG*, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone methyltransferase of *E. coli* (11); *COQ3*, 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase of *S. cerevisiae* (19); *DauC*, aklanonic acid methyltransferase of *Streptomyces* sp. strain C5 (17); *PmtA*, phosphatidylethanolamine methyltransferase of *R. sphaeroides* (18).

An *E. coli* mutant, AN70, is deficient of a specific methylase of the ubiquinone biosynthetic pathway and is completely devoid of MK and contains high levels of demethylmenaquinone (22). We also examined complementation of the growth of AN70 in M9 medium by the expression vector of *o251*.

## EXPERIMENTAL PROCEDURES

*Materials*—[1-$^{14}$C]Isopentenyl diphosphate (IPP; 2.22 GBq/mmol) and *S*-adenosyl-L-[methyl-$^{14}$C]methionine ($^{14}$C-SAM; 2.29 GBq/mmol) were products of Amersham Corp. Nonlabeled IPP and FPP were synthesized according to the procedure of Davisson *et al.* (13). Nonlabeled SAM was purchased from Sigma. Precoated reversed phase TLC plates (LKC-18) were products of Whatman. T4 DNA ligase and DNA polymerase were purchased from Takara Shuzo Co., Ltd. All other chemicals were of analytical grade.

*General Procedures*—Restriction enzyme digestion, transformation, and other standard molecular biology techniques were carried out as described by Sambrook *et al.* (14).

*Bacterial Strains and Media*—*E. coli* K12 strain JM109 was used as the host to express the recombinant proteins. *E. coli* strains containing plasmid vectors conferring ampicillin resistance were maintained on Luria-Bertani medium supplemented with 50 $\mu$g/ml ampicillin. An *E. coli* strain AN70 used for complementation experiments was kindly supplied by Drs. G. Unden and I. Z. Young.

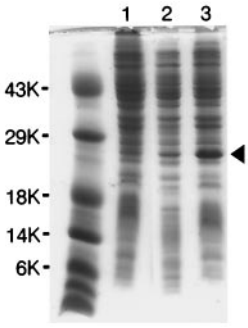


FIG. 3. **Overexpression of the ORF-2 gene.** *E. coli* JM109 harboring pHE84 was incubated without (*lane 2*) or with isopropyl-$\beta$-D-thiogalactopyranoside (*lane 3*). *E. coli* JM109 harboring pTrc99A was incubated as the control (*lane 1*). Total proteins were electrophoresed on SDS-polyacrylamide gel. The protein markers are indicated on the *left*. The ORF-2 gene product is shown by an *arrowhead*.

*Construction of Expression Vector*—The original cloning of the *B. stearothermophilus* HepPP synthase gene cluster, ORF-1 (*heps-1*), ORF-2, and ORF-3 (*heps-2*) in pT7 Blue-T vector as pTL6 was previously described (9). To introduce a *Sph*I site at 5′ end and a *Hin*dIII site at 3′ end of ORF-2, mutagenic oligonucleotide primers, 5′-AAGGGTAGAAGCATGCGTCAATCG-3′ (mismatched bases are underlined) and 5′-CATCGCCTTAAGCTTCATGTTGTTCACC-3′ were synthesized, respectively. Conditions for polymerase chain reaction (PCR) were: 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min, followed by extension at 72°C for 7 min. To a final volume of 100 $\mu$l, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 $\mu$M each of dNTPs, 100 pmol of the amplification primer pair, 1 unit of DNA polymerase enhancer (Stratagene), approximately 1 ng of pTL6, and 2 units of *Taq* polymerase were added. The PCR products were digested with *Sph*I, treated with T4 DNA polymerase, and digested again with *Hin*dIII. The samples were subjected to electrophoresis on a 0.8% agarose gel. Approximately 0.7-kilobase pair fragments were isolated, cloned into pTrc99A vector (Pharmacia Biotech Inc.), which was digested with *Nco*I, treated with T4 DNA polymerase, and digested with *Hin*dIII. The resulting plasmid was designated pHE84.

To introduce an *Nco*I site at 5′ end and a *Hin*dIII site at 3′ end of *o251* of *E. coli*, mutagenic oligonucleotide primers, 5′-GAGCAGGCATTGCCATGGTGG-3′ (mismatched bases are underlined) and 5′-AAAAAGCTTTTCCGGTCTCC-3′ were synthesized. PCR was carried out under the same conditions as described above, using the genomic DNA from *E. coli* as the template. Then the PCR products were subjected to electrophoresis on a 0.8% agarose gel, purified, digested with *Nco*I and *Hin*dIII, and inserted into *Nco*I/*Hin*dIII site of pTrc99A. The resulting plasmid was designated pO251.

*Protein Expression in E. coli cells*—Cells of *E. coli* containing plasmid pHE84 were grown at 37 °C to $A_{600}$ of 0.6 to 0.8, and isopropyl-$\beta$-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce the expression of the ORF-2 product. After induction for 3 h the cells were collected by centrifugation and subjected to electrophoresis according to the standard method of Laemmli (15).

*Preparation of Crude Homogenate of the Cells—E. coli* cells were grown into late exponential phase in Luria-Bertani medium. The cells were centrifuged, suspended in a solution of 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol (2 ml/g of wet cells), and disrupted with a Branson sonifier.

*Methyltransferase Assay and Product Analysis*—The incubation mixture contained, in a total volume of 0.5 ml, 0.2 ml of crude homogenate of *E. coli* cells, 0.1 M Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.4% Triton X-100, 0.5 nmol of FPP, and 0.5 nmol of 1,4-dihydroxy-2-naphthoic acid in 1 $\mu$l of ethanol-diethyl ether (2:1, v/v). In addition, 0.47 nmol of [1-$^{14}$C]IPP with 5 nmol of nonlabeled SAM or 0.5 nmol of nonlabeled IPP with 0.8 nmol of $^{14}$C-SAM were added as substrates in reaction mixtures. After incubation at 37 °C for 1 h, the reaction was stopped by addition of 0.5 ml of 0.1 M acetic acid in methanol. The reaction products were extracted with pentane (2 × 2 ml), analyzed by reversed phase TLC with a solvent system of acetone/

water (19:1). The positions of authentic standards were visualized with iodine vapor, and the distribution of radioactivity was detected by autoradiography with a Fuji BAS 1000 Mac bioimage analyzer.

## RESULTS

*Sequence of the ORF-2 Product*—Homology search revealed that the predicted amino acid sequence of ORF-2 contains the consensus sequence of some methyltransferases that require SAM as substrate (Fig. 2) (16).

DauC (27% identity) of *Streptomyces* sp. strain C5 catalyzes the methyl esterification of aklanonic acid in daunomycin biosynthesis (17). *E. coli* UbiG (22% identity) catalyzes SAM-dependent methylation of 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone, the terminal step in the ubiquinone biosynthesis (11). *Rhodobacter sphaeroides* PmtA (27% identity) catalyzes the *N*-methylation of phosphatidylethanolamine (18). COQ3 (17% identity) catalyzes the transfer of a methyl group from SAM to the 3-hydroxyl of 3,4-dihydroxy-5-hexaprenylbenzoate in ubiquinone biosynthesis (19). There are two unidentified genes, *gerC2* of *B. subtilis* (10) and *o251* of *E. coli* (12), whose predicted protein products showed 69 and 37% identity to the ORF-2 product, respectively.

*Construction of Expression Vector of ORF-2*—To identify the ORF-2 product, the *Nco*I-*Hin*dIII fragment of the PCR product containing ORF-2 was inserted in pTrc99A, and the resulting plasmid was named as pHE84, which was transformed into *E. coli* JM109 for expression. *E. coli* JM109 carrying pHE84 overproduced the ORF-2 product by induction with isopropyl-β-D-thiogalactopyranoside (Fig. 3).

*Methyltransferase Assay*—The crude homogenate of *E. coli* JM109 harboring pHE84, which contained the ORF-2 products overproduced, was incubated with 0.47 nmol of [1-$^{14}$C]IPP with 5 nmol of nonlabeled SAM or 0.5 nmol of nonlabeled IPP with 0.8 nmol of $^{14}$C-SAM. After incubation the products were extracted with pentane and chromatographed on a reversed phase LKC-18 plate with a solvent system of acetone/water (19:1, v/v) (Fig. 4).

Both the autoradiograms of the products derived from the reaction with [1-$^{14}$C]IPP (*lane 4*) and those derived from the reaction with $^{14}$C-SAM (*lane 3*) showed a major radioactivity spot coinciding with MK-8. The radioactivity of the spot of MK-8 increased with the reaction time as shown in Fig. 4*b*. These facts clearly indicate that the homogenate of *E. coli* cells harboring pHE84 shows 2-octaprenyl-1,4-naphthoquinone methyltransferase activity, which transfers a methyl group from SAM to synthesize MK-8.

Another major radioactivity spot coinciding with farnesol in *lane 4* seems to be the product synthesized by endogenous IPP isomerase, FPP synthase, and a phosphatase of the host cells.

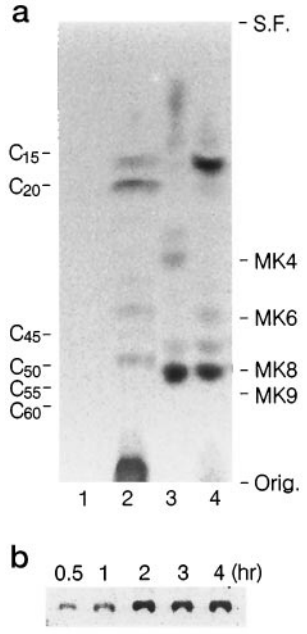


FIG. 4. **TLC radiochromatograms of the pentane extracts of the products formed by the incubation with the homogenate of *E. coli* JM109/pHE84.** *a*, the crude homogenate of *E. coli* JM109 harboring pHE84 was incubated in the mixture as described under "Experimental Procedures" with 0.5 nmol of nonlabeled IPP and 0.8 nmol of $^{14}$C-SAM (*lane 3*) or 0.47 nmol of [1-$^{14}$C]IPP and 5 nmol of nonlabeled SAM (*lane 4*). *E. coli* JM109 harboring pTrc99A was incubated under the same condition with 0.5 nmol of nonlabeled IPP and 0.8 nmol of $^{14}$C-SAM (*lane 1*) or 0.47 nmol of [1-$^{14}$C]IPP and 5 nmol of nonlabeled SAM (*lane 2*). The positions of authentic prenyl alcohols and MKs co-chromatographed are indicated on the *left* and *right lanes*, respectively. *Orig.*, origin; *S.F.*, solvent front. *b*, time course of MK-8 formation in the incubation with 0.5 nmol of nonlabeled IPP and 0.8 nmol of $^{14}$C-SAM.

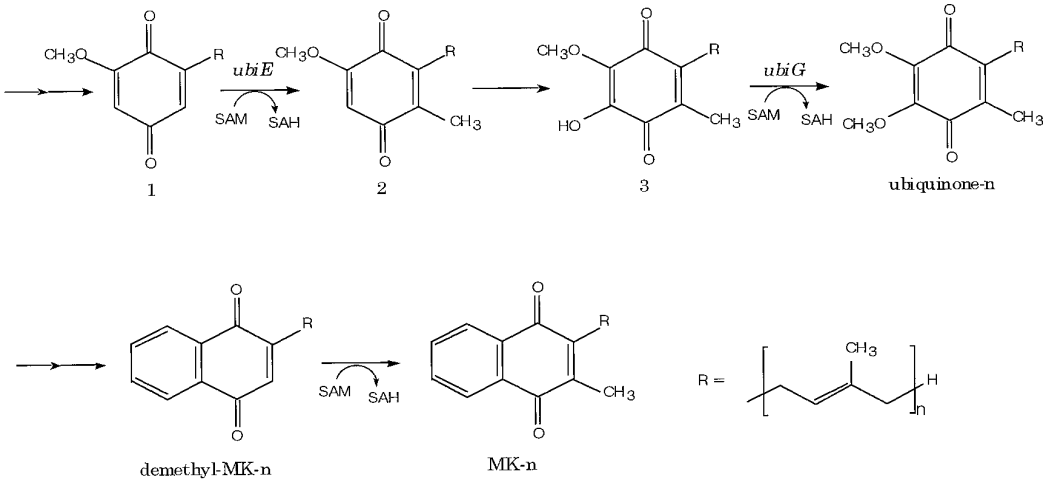


FIG. 5. **Methylation reactions and intermediates in the biosynthetic pathway of MK and ubiquinone.** *Italics* show the genetic loci in mutant strains of each methylation step. The length of the isoprenoid side chain (*n*) varies depending on the species. *SAH*, *S*-adenosylhomocysteine; *1*, 2-polyprenyl-6-methoxy-1,4-benzoquinone; *2*, 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinone; *3*, 2-polyprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone.

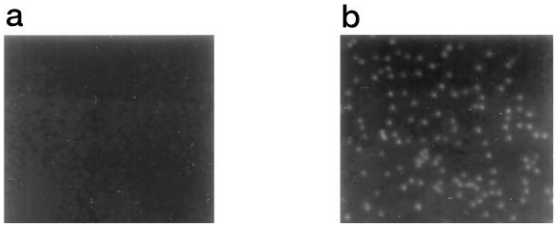


FIG. 6. **Complementation of the *E. coli* mutant AN70.** *a*, AN70. *b*, AN70 harboring pO251 were grown on M9 medium plates at 37 °C.

When a similar incubation was carried out with the addition of purified FPP synthase of *B. stearothermophilus* (20), this spot became more distinct (data not shown).

*Complementation of the E. coli mutant*—The *E. coli* mutant AN70 cells transformed with pO251 were grown on M9 medium plates. The *E. coli* AN70 cells harboring pO251 grew well on an M9 medium plate and formed colonies (see Fig. 6). In contrast, AN70 could not grow on this medium.

## DISCUSSION

The biosynthetic pathway of MKs has been studied in some detail (21). In *B. stearothermophilus* the prenyl side chain of MK-7 is derived from HepPP, which is synthesized by HepPP synthase. The gene region of the HepPP synthase has been previously cloned and sequenced, showing three open reading frames, ORF-1, ORF-2, and ORF-3. The structural genes of HepPP synthase have been identified to be ORF-1 and ORF-3, designated *heps-1* and *heps-2*, respectively (9). ORF-2 encodes a protein with a molecular weight of 27,132, which has similar sequences to those of some methyltransferases. In this study, we directly identified ORF-2 as the gene encoding 2-heptaprenyl-1,4-naphthoquinone methyltransferase by examining the enzymatic activity of its product.

There are at least seven enzymes that are involved in the biosynthesis of MK as shown in Fig. 1. Methylation of 2-heptaprenyl-1,4-naphthoquinone is the final step in the biosynthesis of MK-7. The genes encoding the enzymes for the other five steps in menaquinone biosynthesis have been identified so far, and four of them have been shown to be in a cluster (*menB*, *menC*, *menD*, and *menE*) in *E. coli* (2). A similar cluster has been also found in *B. subtilis* (6), although *menC* has not yet been identified. However, the 2-heptaprenyl-1,4-naphthoquinone methyltransferase gene has not yet been cloned. The ORF-2 of *B. stearothermophilus*, which is identified as the 2-heptaprenyl-1,4-naphthoquinone methyltransferase gene of this bacterium, is located between the genes encoding the two peculiar components of HepPP synthase. Thus we designate this gene *menG*.

Fig. 2 shows the comparison of amino acid sequence between 2-heptaprenyl-1,4-naphthoquinone methyltransferase, MenG of *B. stearothermophilus*, and other methyltransferases. *B. stearothermophilus* 2-heptaprenyl-1,4-naphthoquinone methyltransferase and the unidentified *E. coli* protein encoded by *o251* share a 37% amino acid sequence identity. Daniels *et al.* have indicated that *ubiE* is genetically mapped to the region from 84.5 to 86.5 min on the *E. coli* chromosome, but they have not identified the gene (12). In this region there are 82 open reading frames including *o251*, which is located at 86 min on the *E. coli* chromosome. Of particular interest is the fact that UbiE catalyzes SAM-dependent methylation of 2-octaprenyl-6-methoxy-1,4-benzoquinone, the latter step in the biosynthesis of ubiquinone (21). From the map position and its sequence similarity to that of some methyltransferases, *o251* seems to correspond to *ubiE* (Fig. 5). *E. coli* produces both of the isoprenoid quinones of ubiquinone-8 and MK-8. An *E. coli* mutant AN70 (*ubiE*), which is deficient of a specific methyltransferase in the ubiquinone biosynthetic pathway, has also been shown to be completely devoid of MK and contain a high level of demethylmenaquinone-8 (22). Our complementation experiments with the mutant AN70 harboring pO251, which contains *o251* of *E. coli* in pTrc99A, showed growth restoration of the *E. coli* mutant on a minimal medium (Fig. 6). These results suggest that *E. coli* UbiE was encoded by *o251* in *E. coli*. The methylation of demethyl-MK is chemically very similar to that of 2-octaprenyl-6-methoxy-1,4-benzoquinone catalyzed by UbiE, because in both reactions the methyl group is transferred to the 1,4-quinoid ring systems at position C-3. It is therefore interesting to examine whether *ubiE* encodes the methyltransferase that catalyzes the transfer of methyl groups both to 2-octaprenyl-6-methoxy-1,4-benzoquinone and to 2-octaprenyl-1,4-naphthoquinone.

Because MK-7 is the dominant MK in *B. stearothermophilus*, the ORF-2 product should be assigned as 2-heptaprenyl-1,4-naphthoquinone methyltransferase. Thus, we propose to designate this gene *menG*. In our *in vitro* experiments the cell homogenate of *E. coli* harboring pHE84 synthesized MK-8, which has the prenyl side chain one isoprene unit longer than that of *B. stearothermophilus*. This fact indicates that the substrate specificity of the methyltransferase is not very stringent for the prenyl side chain of demethyl-MK. This is similar to the specificities of UbiA of *E. coli* (23) and of COQ2 of *Saccharomyces cerevisiae* (24), which are 4-hydroxy-benzoate-octaprenyl transferase and 4-hydroxy-benzoate-hexaprenyl transferase in ubiquinone biosynthesis, respectively. Similarly, the 1,4-dihydroxy-2-naphthoate-octaprenyl transferase of *Micrococcus luteus* has been shown to have such a wide specificity with respect to prenyl diphosphate substrates (25).

*Acknowledgments*—We are grateful to Dr. G. Unden and Dr. I. G. Young for suppling the bacterial strain AN70 and Chika Ishida for cooperation in the construction of pO251.

## REFERENCES

1. Farrand, S. K., and Taber, H. W. (1973) *J. Bacteriol.* **115,** 1035–1044
2. Guest, J. R., and Shaw, D. J. (1981) *Mol. & Gen. Genet.* **181,** 379–383
3. Sharma, V., Suvarna, K., Meganathan, R., and Hudspeth, M. E. (1992) *J. Bacteriol.* **174,** 5057–5062
4. Sharma, V., Meganathan, R., and Hudspeth, M. E. (1993) *J. Bacteriol.* **175,** 4917–4921
5. Palaniappan, C., Sharma, V., Hudspeth, M. E., and Meganathan, R. (1992) *J. Bacteriol.* **174,** 8111–8118
6. Miller, P., Rabinowitz, A., and Taber, H. (1988) *J. Bacteriol.* **170,** 2735–2741
7. Driscoll, J. R., and Taber, H. W. (1992) *J. Bacteriol.* **174,** 5063–5071
8. Rowland, B., Hill, K., Miller, P., Driscoll, J., and Taber, H. (1995) *Gene* (*Amst.*) **167,** 105–109
9. Koike-Takeshita, A., Koyama, T., Obata, S., and Ogura, K. (1995) *J. Biol. Chem.* **270,** 18396–18400
10. Yazdi, M. A., and Moir, A. (1990) *J. Gen. Microbiol.* **136,** 1335–1342
11. Wu, G., Williams, H. D., Zamanian, M., Gibson, F., and Poole, R. K. (1992) *J. Gen. Microbiol.* **138,** 2101–2112
12. Daniels, D. L., Plunkett, G. 3., Burland, V., and Blattner, F. R. (1992) *Science* **257,** 771–778
13. Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M., and Poulter, C. D. (1986) *J. Org. Chem.* **51,** 4768–4779
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Laemmli, U. K. (1970) *Nature* **227,** 680–685
16. Ingrosso, D., Fowler, A. V., Bleibaum, J., and Clarke, S. (1989) *J. Biol. Chem.* **264,** 20131–20139
17. Dickens, M. L., Ye, J., and Strohl, W. R. (1995) *J. Bacteriol.* **177,** 536–543
18. Arondel, V., Benning, C., and Somerville, C. R. (1993) *J. Biol. Chem.* **268,** 16002–16008
19. Clarke, C. F., Williams, W., and Teruya, J. H. (1991) *J. Biol. Chem.* **266,** 16636–16644
20. Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchida, M., Nishino, T., and Ogura, K. (1993) *J. Biochem.* (*Tokyo*) **113,** 355–363
21. Bentley, R., and Meganathan, R. (1987) in *Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds) pp. 512–520, American Society for Microbiology, Washington, D.C.
22. Wissenbach, U., Ternes, D., and Unden, G. (1992) *Arch. Microbiol.* **158,** 68–73
23. Melzer, M., and Heide, L. (1994) *Biochim. Biophys. Acta* **1212,** 93–102
24. Suzuki, K., Ueda, M., Yuasa, M., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1994) *Biosci. Biotechnol. Biochem.* **58,** 1814–1819
25. Saito, Y., and Ogura, K. (1981) *J. Biochem.* (*Tokyo*) **89,** 1445–1452


CARBOHYDRATES, LIPIDS, AND OTHER NATURAL PRODUCTS:

# Identification of a Novel Gene Cluster Participating in Menaquinone (Vitamin $K_2$) Biosynthesis: CLONING AND SEQUENCE DETERMINATION OF THE 2-HEPTAPRENYL-1,4-NAPHTHOQUINONE METHYLTRANSFERASE GENE OFBACILLUS STEAROTHERMOPHILUS

Ayumi Koike-Takeshita, Tanetoshi Koyama and Kyozo Ogura

*J. Biol. Chem. 1997, 272:12380-12383.*
*doi: 10.1074/jbc.272.19.12380*