J. Biochem. **124**, 790-797 (1998)

Intersubunit Structure within Heterodimers of Medium-Chain Prenyl Diphosphate Synthases. Formation of a Hybrid-Type Heptaprenyl Diphosphate Synthase[1]

Ayumi Koike-Takeshita,* Tanetoshi Koyama,†,[2] and Kyozo Ogura†

**Bio Research Laboratory, Toyota Motor Corporation, Toyota-cho 1, Toyota, Aichi 471-0826; and* †*Institute for Chemical Reaction Science, Tohoku University, Aoba-ku, Sendai, Miyagi 980-8577*

Received for publication, May 25, 1998

Among prenyltransferases that catalyze the sequential condensation of isopentenyl diphosphate with allylic diphosphate to produce prenyl diphosphates with various chain lengths and stereochemistries, medium-chain prenyl diphosphate synthases are exceptional in that they comprise two dissociable heteromeric protein components. These components exist without binding with each other under physiological conditions, and neither of them has any prenyltransferase activity by itself. In order to elucidate the precise molecular mechanism underlying expression of the catalytic function by such a unique two-component system, we examined the possibility of forming a hybrid between two of the components of three different medium-chain prenyl diphosphate synthases, components I and II of heptaprenyl diphosphate synthase from *Bacillus subtilis*, components I′ and II′ of heptaprenyl diphosphate synthase from *Bacillus stearothermophilus*, and components A and B of hexaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26. As a result, only the hybrid-type combination of component I and component II′ gave distinct prenyltransferase activity. The hybrid-type enzyme catalyzed the synthesis of heptaprenyl diphosphate and showed moderate heat stability, which lay between those of the natural enzymes from *B. subtilis* and *B. stearothermophilus*. There is no possibility of forming a hybrid between the heptaprenyl and hexaprenyl diphosphate synthases.

Key words: heteromeric components, isoprenoid biosynthesis, prenyl diphosphate, prenyltransferase, subunit association.

Prenyltransferases catalyze fundamental isoprenoid chain elongation to produce prenyl diphosphates with various chain lengths and stereochemistries, which are converted to such diverse isoprenoid compounds as steroids, carotenoids, glycosyl carrier lipids, prenyl quinones, and prenyl proteins (*1, 2*). These enzymes can be classified into four groups according to the mode of requirement for enzymatic activity (*3*). Short-chain prenyl diphosphate synthases, such as farnesyl- (FPP) (*4*) and geranylgeranyl (*5*) diphosphate (GGPP) synthases, require no cofactor except divalent metal ions such as Mg^{2+} or Mn^{2+}, which are commonly required by all prenyltransferases. The enzymes that catalyze the formation of (*Z*)-polyprenyl chains, including nonaprenyl- (*6*), undecaprenyl- (*7*), and dehydrodolichyl- (*8*) diphosphate synthases, require a phospholipid or detergent. The enzymes catalyzing the syntheses of long-chain (*E*)-prenyl diphosphates, including octaprenyl- (C_{40}), solanesyl- (C_{45}, all-*E*-nonaprenyl-), and decaprenyl- (C_{50}) diphosphate synthases, require protein factors that remove polyprenyl products from their active sites to facilitate and maintain turnover of catalysis (*9*). All of these enzymes are tightly bound homodimers.

On the other hand, hexaprenyl (C_{30}) diphosphate (HexPP) synthase from *Micrococcus luteus* B-P 26 (*10-12*) and heptaprenyl (C_{35}) diphosphate (HepPP) synthase from *Bacillus subtilis* (*13, 14*), which catalyze the synthesis of medium-chain (*E*)-prenyl diphosphates, are peculiar because they do not require a lipid or detergent and comprise two non-identical protein components, designated as components A and B, and components I and II, respectively. These components exist without binding with each other under physiological conditions, and neither of them has any catalytic activity by itself. The catalytic activity can be expressed only in the presence of the counterpart.

Recently, the genes of HexPP synthase of *Saccharomyces cerevisiae* (*15*), HepPP synthase of *B. stearothermophilus* (*3*), and octaprenyl diphosphate synthase of *Escherichia coli* (*16*) were cloned. However, only the HepPP synthase was found to comprise of two heteromeric components (components I′ and II′). Comparison of the deduced primary structures revealed that component II′ of the HepPP synthase shows high similarity with other prenyltransferases, such as many FPP synthases (*17-20*) and GGPP synthases (*21-23*), which are tightly bound homodimers.

[1] This work was supported by Grants-in-Aid for Scientific Research 06240102 and 09480138, from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed: Tel: +81-22-217-5621, Fax: +81-22-217-5620, E-mail: koyama@icrs.tohoku.ac.jp

Abbreviations: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; HepPP, heptaprenyl diphosphate; HexPP, hexaprenyl diphosphate; IPP, isopentenyl diphosphate; IPTG, isopropyl-β-thiogalactopyranoside.

© 1998 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

Our question is why the HepPP synthase comprises two non-identical dissociable protein components, but catalyzes similar condensation reactions. What are the roles of these components?

In the meantime we cloned the genes encoding the two components of HexPP synthase of *M. luteus* B-P 26 (*24*). We also identified the two genes, *gerC1* and *gerC3*, as the structural genes for the two components of HepPP synthase from *B. subtilis* (*25*). Then, we examined the possibility of the formation of an active hybrid enzyme from the components of these medium-chain prenyl diphosphate synthases, *i.e.* HepPP synthases of *B. stearothermophilus* (*3*) and *B. subtilis*, and HexPP synthase of *M. luteus* B-P 26. This paper reports the finding that a hybrid-type enzyme, composed of component I of *B. subtilis* HepPP synthase and component II′ of *B. stearothermophilus* HepPP synthase, can be formed due to the interchangeability of the separable components and shows distinct HepPP synthase activity.

EXPERIMENTAL PROCEDURES

Materials—[1-^{14}C]Isopentenyl diphosphate (IPP; 2.22 GBq/mmol) was a product of Amersham. Nonlabeled IPP and (*E,E*)-FPP were synthesized according to the procedure of Davisson *et al.* (*26*). Precoated reversed phase TLC plates (LKC-18) were products of Whatman. T4 DNA ligase and DNA polymerase were purchased from Takara Shuzo, Kyoto. 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.* (*27*).

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 (LB) medium and M9YG medium (*19*) supplemented with 50 μg/ml ampicillin.

Construction of an 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 the pT7 Blue-T vector as pTL6 was previously described (*3*). Because we detected several point mutations attributable to the PCR, two positions in ORF-1 and one in ORF-2 in the inserted fragment of pTL6, the *Xho*I fragment (1,198 bp) of pTL6 was exchanged with the corresponding *Xho*I fragment of pAC2 (*3*), and the resulting plasmid was designated as pTL7. After deletion of the *Sac*I fragment (1,027 bp) from pTL7, the residual DNA fragment was ligated, and the resulting plasmid was designated as pTLD18, which was equivalent with the correct sequence to pTLD7 in our previous report (*3*).

An expression vector containing *heps-2* of *B. stearothermophilus* was constructed as follows. To introduce a *Bsp*HI site at the 5′ end and a *Hin*dIII site at the 3′ end of ORF-2, mutagenic oligonucleotide primers, 5′-GGTGAACATCATGAAGTTAAAGGCG-3′ (mismatched base is underlined) and 5′-CGTCCTTGAAAGCTTTAATAATCCC-3′, were synthesized, respectively. The conditions for 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 final extension at 72°C for 7 min. To a final volume of 100 μl, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each dNTP, 100 pmol of an 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 *Bsp*HI and *Hin*dIII. Samples were subjected to electrophoresis on a 0.8% agarose gel. Approximately 1 kbp fragments were isolated and inserted into the *Nco*I-*Hin*dIII site of the pTrc99A vector (Pharmacia). The resulting plasmid was designated as pHE5.

Protein Expression in E. coli Cells—*E. coli* cells containing the expression plasmids were grown at 37°C to an A_{600} of 0.6 to 0.8, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the gene constructs. After induction for 3 h the cells were collected by centrifugation and used for each experiment. SDS-PAGE was performed with a discontinuous buffer system (*28*), and the gels were stained with Coomassie Brilliant Blue R.

Preparation of Antiserum against the Recombinant heps-2 Products—*E. coli* JM109 cells (24 g) harboring pHE5 were harvested from a 10-liter culture in LB medium by centrifugation, suspended in 30 ml of 25 mM Tris-HCl buffer, pH 7.0, containing 1 mM EDTA and 10 mM 2-mercaptoethanol, and then disrupted at 0°C for 10 min with a Branson sonifier. After centrifugation at 10,000 × *g* for 30 min, the supernatant was heated at 55°C for 20 min and then centrifuged at 10,000 × *g* for 30 min. The supernatant was chromatographed on a HiTrap SP column (15 ml, Pharmacia) equilibrated with 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Elution was performed with a linear gradient of 0-1 M NaCl. Fractions containing the *heps-2* product were collected and then subjected to SDS-PAGE. The protein in the approximately 36 kDa band was eluted from an acrylamide gel slice with the electrophoresis buffer. Rabbit antiserum against the recombinant *heps-2* product was prepared by Medical & Biological Laboratories, Nagoya.

Preparation of Crude Homogenates of the Cells—*E. coli* cells harboring each of pREG1 and pREG3S, which are the expression plasmids for components A and B of HexPP synthase of *M. luteus* B-P 26, respectively (*24*), and each of pTLD18 and pHE5, which are the expression vectors for *heps-1* and *heps-2* from *B. stearothermophilus*, respectively, were grown in LB medium. *E. coli* cells harboring each of pEHA1 and pEHA3 (*25*), which are the expression vectors including *gerC1* and *gerC3* from *B. subtilis*, respectively, were grown in M9YG medium. The cells were centrifuged and suspended in a solution of 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol. The cells were disrupted with a Branson sonifier. After centrifugation at 10,000 × *g* for 30 min, only the supernatant of *E. coli* cells harboring the expression vectors including each of the *B. stearothermophilus* HepPP synthase genes, *heps-1* and *heps-2*, was heated at 55°C for 20 min and then centrifuged at 10,000 × *g* for 30 min.

Prenyltransferase Assay and Product Analysis—The incubation mixture contained, in a total volume of 250 μl, the indicated amount of a cell-free extract, 50 mM Tris-HCl buffer, pH 8.0, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 50 mM NH_4Cl, 6.25 nmol of FPP, and 0.1175 nmol of [1-^{14}C]IPP. Incubation was carried out at the indicated temperature for 1 h, and then the reaction mixture was

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

treated with 1-butanol to extract the product of the prenyltransferase reaction. The radioactivity in the butanol extract was measured with an Aloka LSC-1000 liquid scintillation counter.

The radioactive prenyl diphosphate was hydrolyzed to the corresponding alcohol with potato acid phosphatase, and then analyzed by TLC on reversed phase LKC-18 according to the method we reported previously (*29*).

Gel Filtration Analysis of Heptaprenyl Diphosphate Synthase—*E. coli* JM109 cells (50 g) harboring pTL7 were harvested from a 32-liter culture in LB medium by centrifugation. The cells were suspended in 50 ml of 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol, and then disrupted at 0°C for 10 min with a Branson sonifier. After centrifugation at 10,000 × *g* for 30 min, the supernatant was heated at 55°C for 20 min and then centrifuged at 10,000 × *g* for 30 min. The supernatant was chromatographed on a HiLoad 26/10 Q Sepharose HP column (2.6 × 10 cm, Pharmacia) equilibrated with 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Elution was performed with a linear gradient of 0-2 M NaCl. HepPP synthase fractions were collected and then applied to a HiLoad 16/10 phenyl-Sepharose HP column (1.6 × 10 cm, Pharmacia) equilibrated with 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and ammonium sulfate at 28% saturation. Elution was performed with a decreasing linear gradient, from 28 to 0% saturation, of ammonium sulfate in 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol. HepPP synthase fractions were collected and then applied to a Mono Q HR 5/5 column (0.5 × 5 cm, Pharmacia) equilibrated with 25 mM Tris-HCl buffer, pH 7.7, containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Elution was performed with a linear gradient of 0-0.6 M NaCl. The partially purified HepPP synthase fractions were collected and subjected to gel filtration analysis on a Superose 12 HR 10/30 column (1.0 × 30 cm, Pharmacia).

The crude homogenates containing the *gerC1* product (component I of *B. subtilis*; designated as component I-sub, 100 μg protein) and the *heps-2* product (component II′ of *B. stearothermophilus*; component II′-ste, 200 μg protein) were mixed and then incubated separately at 4°C for 30 min in the presence of 25 μM IPP and 1 mM $MgCl_2$, 25 μM FPP and 1 mM $MgCl_2$, or 25 μM IPP, 25 μM FPP and 1 mM $MgCl_2$. Then each of the mixtures was subjected to gel filtration analysis on a Superose 12 column (1 × 30 cm), which had been equilibrated with 50 mM Tris-HCl buffer, pH 7.7, containing 10 mM 2-mercaptoethanol and the same concentrations of the substrate and $MgCl_2$ as in each protein mixtures. Elution was performed with the same buffer system for each equilibration at the flow rate of 0.2 ml/min. Fractions (0.1 ml) were collected, and 10 μl aliquots were assayed for prenyltransferase activity.

Immunoblot Analyses—SDS-PAGE was performed according to the method of Laemmli (*28*). The enzyme solutions for PAGE were prepared in two ways. (a) Reductive conditions: The sample solution comprised 62.5 mM Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, and 0.0005% bromophenol blue. The solution was heated in a boiling water bath for 5 min. (b) Non-reductive conditions: A solution with the same constituents as described above, except that 2-mercaptoethanol was omitted, was applied to the gel without heating. Proteins were transferred to Immobilon-P transfer membranes (Millipore) at 0.8 mA/cm² for 1 h using a Semi-Dry system according to the protocol of Millipore. The blotted membranes were soaked in deionized water for 5 min and then dried at room temperature for over 12 h. The membranes were incubated for 1 h with anti-component II′-ste serum diluted in 20 mM Tris-HCl buffer (pH 7.5), containing 500 mM NaCl, 0.05% Tween 20, and 1% BSA (TBS), and then washed three times for 5 min in 20 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl and 0.05% Tween 20 (TTBS). After washing, the membranes were incubated for 1 h with colloidal gold-labeled goat anti-rabbit IgG (Bio-Rad) diluted 1:25 in 20 mM Tris-HCl buffer (pH 9.0) containing 150 mM NaCl, 0.05% Tween 20, and 1% BSA. The membranes were washed twice in TTBS for 5 min, once in TBS for 5 min, and then twice in deionized water for 1 min. The membranes stained with colloidal gold were assayed according to the protocol of Bio-Rad for the gold enhancement procedure.

RESULTS

Construction of an Expression Vector for the heps-2 Product—The *Bsp*HI-*Hin*dIII fragment of the PCR product containing *heps-2* was cloned to pTrc99A and the resulting plasmid, pHE5, was transformed into *E. coli* JM109 for expression. *E. coli* cells carrying pHE5 overproduced the *heps-2* product (component II′-ste) on induction with IPTG. The protein migrated to a position corresponding to 36 kDa on SDS-PAGE (Fig. 1), which coincided with that predicted from the nucleotide sequence of *heps-2*.

Formation of Hybrid Enzymes—In order to determine whether or not each of the two components is interchangeable with the corresponding counterpart of other enzymes *in vitro*, we examined the formation of catalytically active hybrid enzymes comprising components of the enzymes from different sources. *E. coli* cells harboring pREG1 or pREG3S (*24*) were grown in LB medium separately, and crude homogenates containing the *hexs-a* product (Component A) or *hexs-b* product (Component B) were prepared. *E. coli* cells harboring pEHA1 and pEHA3 (*25*) were used

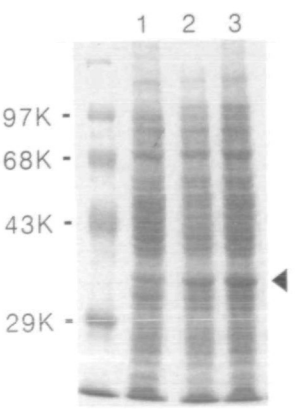

Fig. 1. **Overproduction of the *heps-2* product.** *E. coli* JM109 harboring pHE5 was incubated without (lane 2) or with IPTG (lane 3). *E. coli* JM109 harboring pTrc99A (lane 1). Total proteins were subjected to SDS-PAGE. The protein marker are indicated on the left and the arrowhead shows the *heps-2* product.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

for the preparation of crude homogenates containing the *gerC1* product (Component I-sub) and *gerC3* product (Component II-sub), respectively. Crude homogenates containing the *heps-1* product (Component I′-ste) and *heps-2* product (Component II′-ste) were prepared from *E. coli* cells harboring pTLD18 and pHE5, respectively, and then heated at 55°C for 20 min. We examined all 9 possible combinations of between the two components of three enzymes. As a result, only three combinations, *i.e.* the natural combinations of components I-sub and II-sub of *B. subtilis* HepPP synthase, and components A and B of *M. luteus* B-P 26 HexPP synthase, and a hybrid-type combination of component I-sub of *B. subtilis* and component II′-ste of *B. stearothermophilus*, showed prenyltransferase activity (Table I). The hybrid-type combination of components I-sub and II′-ste was found to produce HepPP, as shown in Fig. 2. The combination of components I′-ste and II′-ste of *B. stearothermophilus* HepPP synthase, both of which were produced in *E. coli* cells separately, did not show any prenyltransferase activity at all. However, when they were coproduced in *E. coli* cells, they showed sufficient HepPP synthase activity.

The dependency of the HepPP synthase activity on the concentration of component I or component II was examined. Both the components of the hybrid-type enzyme showed typical saturation-curves as to the counterpart component (Fig. 3, b and c), as observed with the natural combination of *B. subtilis* HepPP synthase (Fig. 3a). The concentration ratio of component I-sub:component II-sub: component II′-ste was calculated to be approximately 1:0.5:1 from the densities on Coomassie Brilliant Blue staining after SDS-PAGE by use of NIH image. Thus, the optimum ratio of the two components at the saturation point was estimated to be approximately 2 for both combinations, *i.e.* component I-sub plus component II-sub and component I-sub plus component II′-ste. It is of interest that the hybrid-type enzyme showed as much enzymatic activity as the natural enzyme of *B. subtilis* did (Fig. 3).

Thermostability of the Hybrid-Type Enzyme—We ex-

TABLE I. **Assaying of prenyltransferase activity of various combinations of the components of HexPP and HepPP synthases.**

Combination	Activity (dpm)
Component I-sub + Component II-sub	140
Component I-sub + Component II′-ste	7,900
Component I-sub + Component B	0
Component I′-ste + Component II-sub	0
Component I′-ste + Component II′-ste	0
Component I′-ste + Component B	0
Component A + Component II-sub	0
Component A + Component II′-ste	0
Component A + Component B	6,300

The amounts of protein used for this assay were 1 μg of each crude homogenate. The reactions were carried out at 37°C for 1 h.

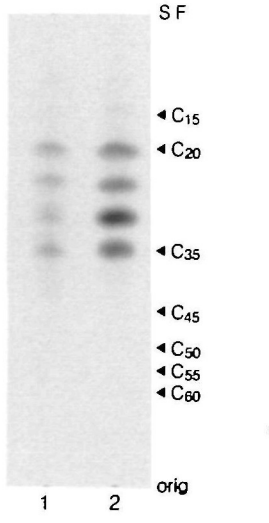

Fig. 2. **TLC radiochromatogram of a pentane extract of the product formed on incubation with the combination of component I-sub and component II-sub or component I-sub and component II′-ste.** The crude homogenate with the combination of component I-sub and component II-sub (lane 1) or component I-sub and component II′-ste (lane 2) was incubated separately as described under "EXPERIMENTAL PROCEDURES" at 37°C for 1 h. The positions of authentic prenyl alcohols are indicated on the right lane. orig., origin; S.F., solvent front.

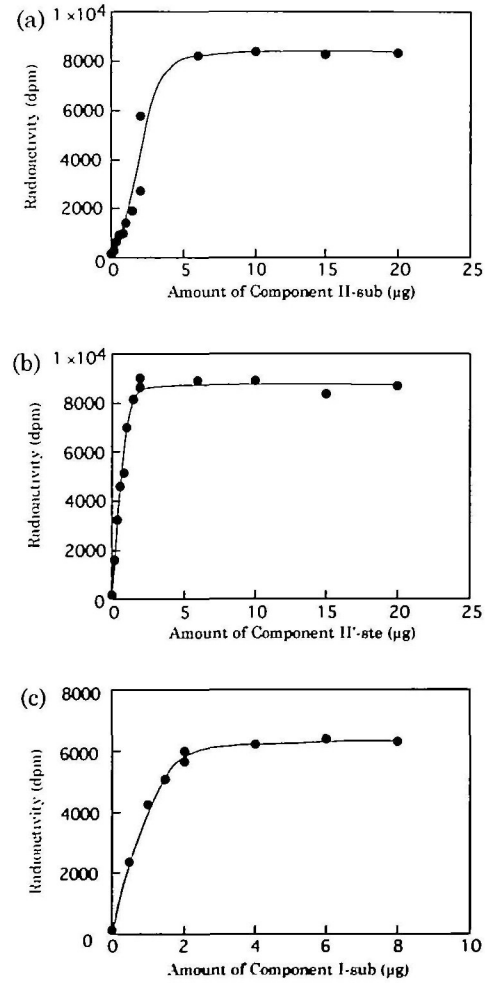

Fig. 3. **Effects of the concentrations of components on enzymatic activity.** A crude homogenate of component I-sub (1 μg) was incubated as described under "EXPERIMENTAL PROCEDURES" at 37°C for 1 h in the presence of various concentrations of a crude homogenate of component II-sub (panel a) or component II′-ste (panel b). A homogenate of component II′-ste (1 μg) was incubated similarly in the presence of various concentrations of a homogenate of component I-sub (panel c).

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

amined the thermostability of the hybrid-type enzyme comprising component I-sub from a mesophilic bacterium and component II′-ste from the thermophilic bacterium. As shown in Fig. 4, the hybrid-type enzyme showed moderate thermostability that lay between those of the natural enzymes of *B. subtilis* and of *B. stearothermophilus*. Although the *B. subtilis* HepPP synthase (component I-sub

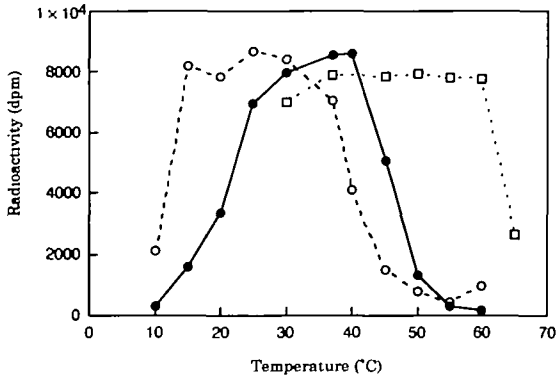

Fig. 4. **Effect of the reaction temperature on enzymatic activity.** A crude homogenate with the combination of component I-sub and component II-sub (○), or component I-sub and component II′-ste (●), and partially purified HepPP synthase from *E. coli* JM109 harboring pTL7 (□) was incubated as described under "EXPERIMENTAL PROCEDURES" at various temperatures for 1 h.

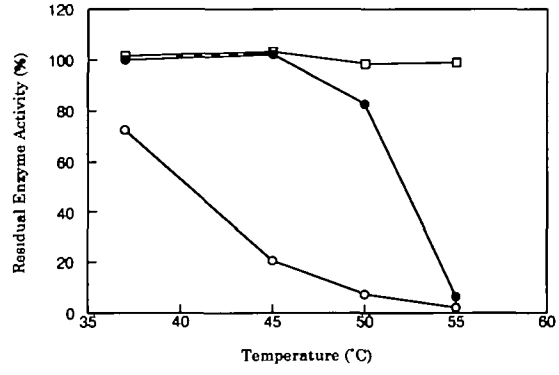

Fig. 5. **Effect of heat treatment.** A crude homogenate with the combination of component I-sub and component II-sub (A) (○), or component I-sub and component II′- ste (B) (●), and partially purified HepPP synthase from *E. coli* JM109 harboring pTL7 (C) (□) was incubated at the indicated temperatures for 20 min, or not incubated, and then assayed as to restortion of the activity of prenyl diphosphate synthase at 30°C (A, B) or 55°C (C) for 1 h.

Fig. 6. **Gel filtration of a mixture of component I-sub and component II′-ste or partially purified *B. stearothermophilus* HepPP synthase (component I′-ste and II′-ste) from recombinant *E. coli*.** A mixture of the homogenate of component I-sub and component II′-ste was chromatographed on a Superose 12 column with various eluents, containing FPP and $MgCl_2$, IPP and $MgCl_2$ (similar elution, B), or FPP, IPP, and $MgCl_2$ (C), or none of them (A), as described under "EXPERIMENTAL PROCEDURES." Partially purified *B. stearothermophilus* HepPP synthase from *E. coli* JM109 harboring pTL7, which included *heps-1* and *heps-2*, was chromatographed in the same way and showed similar elution under all conditions (D). Enzyme activity was assayed in the presence of component I-sub (○) or component II′-ste (□), and in their absence (●). The molecular mass standards were as follows: 1, aldolase (158 kDa); 2, bovine serum albumin (67 kDa); 3, ovalbumin (43 kDa); 4, chymotrypsinogen A (25 kDa).

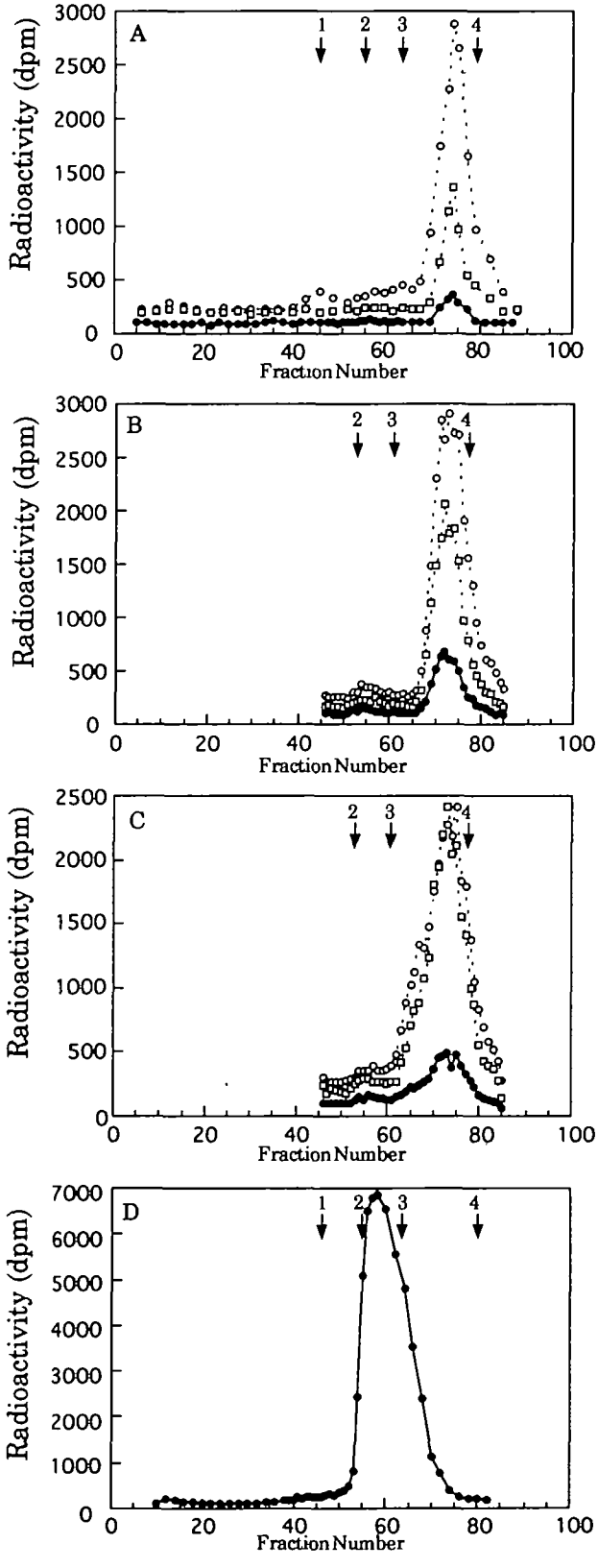

Fig. 6

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

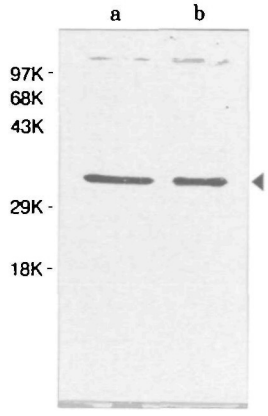

Fig. 7. **SDS-PAGE analysis of partially purified HepPP synthase treated under reductive or non-reductive conditions.** Partially purified HepPP synthase from *E. coli* JM109 harboring pTL7 was analyzed by SDS-PAGE under reductive or non-reductive conditions as described under "EXPERIMENTAL PROCEDURES," and then detected by immunoblotting using antiserum against the recombinant *heps-2* product (component II′-ste). (a) Reductive conditions, (b) Non-reductive conditions.

and component II-sub) showed only 20% remaining activity after heat treatment at 45°C, the hybrid-type showed 83% remaining activity after heating at 50°C (Fig. 5).

Gel Filtration Analyses—HepPP synthase of *B. stearothermophilus* partially purified from *E. coli* cells harboring pTL7, which included both *heps-1* and *heps-2*, and the hybrid-type enzyme, which was a 1:2 mixture of component I-sub and component II′-ste, were subjected to gel filtration separately with various eluents, comprising IPP and $MgCl_2$, FPP and $MgCl_2$, and IPP, FPP, and $MgCl_2$. As shown in Fig. 6, *B. stearothermophilus* HepPP synthase (components I′-ste and II′-ste) was eluted at a position corresponding to a molecular mass of 60 kDa under all employed elution conditions (panel D). This value is in approximate accord with the sum of the predicted molecular mass values of Heps-1 and Heps-2. On the other hand, the hybrid-type enzyme showed an elution position corresponding to 30 kDa in the absence of $MgCl_2$ and a substrate (panel A), and an elution position corresponding to 30 kDa and a very small elution peak at 60 kDa in the presence of IPP and $MgCl_2$ or FPP and $MgCl_2$ (panel B). As shown in panel C, gel filtration of the hybrid-type enzyme in the presence of IPP, FPP, and $MgCl_2$ gave a broad elution peak ranging from 60 to 30 kDa with a top at 30 kDa. These results indicate that component I-sub and component II′-ste are dissociated in the absence of a substrate, and weakly interact in the presence of a substrate.

Subunit Interaction Analysis of B. stearothermophilus HepPP Synthase—In order to investigate the interaction between the components of *B. stearothermophilus* HepPP synthase, SDS-PAGE was performed under reductive or non-reductive conditions. As a result, on immunoblotting, the antiserum against the recombinant component II′-ste gave a 36-kDa protein band on SDS-PAGE under both conditions (Fig. 7). This finding shows that the components of *B. stearothermophilus* HepPP synthase are dissociable from each other even on SDS under nonreductive conditions, suggesting a hydrophobic interaction.

DISCUSSION

We previously cloned and sequenced the structural genes of *B. stearothermophilus* HepPP synthase, *heps-1* and *heps-2*, which encode predicted translation products with molecular weights of 25 and 36 kDa, respectively (*3*). The HepPP synthase from *B. subtilis* (*13, 14*) and the HexPP synthase from *M. luteus* B-P 26 (*10–12*) are unique in that each of them comprises two dissociable protein components, neither of which shows any enzyme activity. Recently, the structural genes of these enzymes were cloned and sequenced (*24, 25*). The structural genes of the HepPP synthase from *B. subtilis*, *gerC1* and *gerC3*, encode predicted translation products with molecular weights of 29 and 36 kDa, respectively (*25*), and those of the HexPP synthase from *M. luteus* B-P 26, *hexs-a* and *hexs-b*, encode predicted translation products with molecular weights of 17 and 37 kDa, respectively (*24*). Heps-1 (component I′-ste) shows 30% identity to GerC1 (component I-sub) and is 8.2% identity to Hexs-a (component A), which is about 100 amino acids shorter than Heps-1 or GerC1 in the carboxyl terminal region. Heps-2 (component II′-ste) shows 64% identity to GerC3 (component II-sub) and 36% identity to Hexs-b (component B).

We examined the possibility of the formation of a catalytically active hybrid-type enzyme composed of components of the enzymes from three different sources. As a result, only one combination, component I of *B. subtilis* (component I-sub) and component II′ of *B. stearothermophilus* (component II′-ste), showed prenyltransferase activity and moderate thermostability that lay between those of *B. subtilis* and *B. stearothermophilus*. This result shows the interchangeability of components I′-ste and I-sub even though they only show 38% identity. It was previously reported that component II was less stable as to heat treatment than component I in the HepPP synthase from *B. subtilis* (*13*). As unstable component II-sub was substituted for thermostable component II′-ste, it seems reasonable that the hybrid-type enzyme became more thermostable than the natural HepPP synthase of *B. subtilis*. Although the combination of components I′ and -II′ from *B. stearothermophilus*, both of which were produced in *E. coli* cells separately, did not show any prenyltransferase activity, the hybrid-type enzyme expressed sufficient HepPP synthase activity, like the natural combination of components I and II from *B. subtilis* did. All our efforts to overproduce component I′ of *B. stearothermophilus* using various expression vectors resulted in the formation of inactive inclusion bodies (data not shown). Comparison of the hydrophobicities of component I of *B. subtilis* and component I′ of *B. stearothermophilus* is shown in Fig. 8. Although the hydrophobicity profile for the whole sequence of component I′ of HepPP synthase from *B. stearothermophilus* is similar to that of component I from *B. subtilis*, component I′-ste is more hydrophobic than component I-sub (Fig. 8). It seems likely that component I′-ste can not be folded correctly because of its high hydrophobicity unless it is coproduced with its counterpart, component II′-ste. While Hexs-b (component B) showed 36 and 41% identity to Heps-2 (component II′-ste) and GerC3 (component II-sub), respectively, the formation of a catalytically active hybrid-type enzyme was not observed between HexPP synthase

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

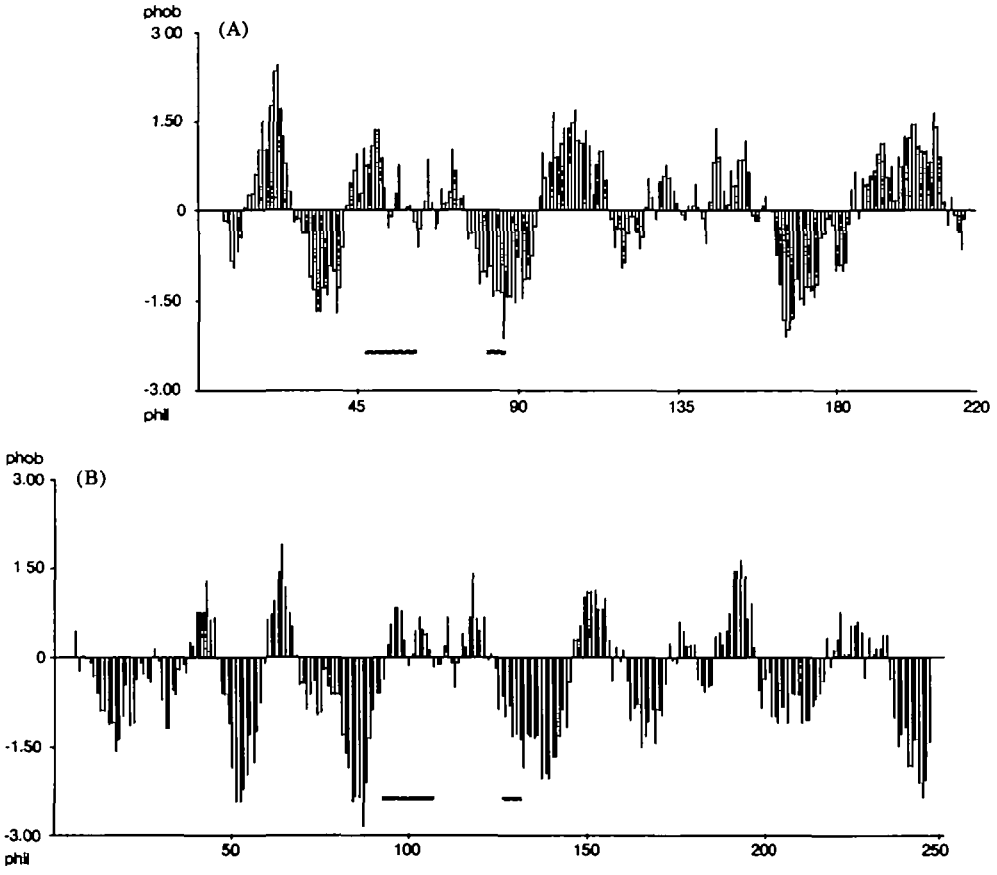

Fig. 8. **Hydrophobicity profiles for the primary sequences of component I-sub and component I′-ste.** The amino acids are numbered. Positive values represent hydrophobic amino acids and negative values indicate hydrophilic residues. The consensus sequences in component I of HepPP synthase and component A of HexPP synthase suggested by Shimizu *et al.* (*24*) are represented by bars. (A) component I′-ste, (B) component I-sub.

and HepPP synthase. Even the formation of a heteromeric dimer between the components of HexPP- and HepPP synthases might be impossible because of the low identity (approximately 8%) between Hexs-a (component A) and Heps-1 (component I′-ste) or Ger C1 (component I-sub).

It has been reported that the two components of HexPP synthase (components A and B) of *M. luteus* B-P 26 exist as dissociated forms under physiological conditions, and form a complex of the two components in the presence of a substrate (*11, 12*). Recently, Zhang *et al.* demonstrated in detail that components I and II of HepPP synthase of *B. subtilis* form a catalytically active 1:1 complex of 66 kDa in the presence of FPP and Mg^{2+} (*30*). However, neither the two components of the native *B. stearothermophilus* HepPP synthase (*31*) nor those of the recombinant enzyme from *E. coli* cells harboring pTL7 was found to be dissociable under any chromatographic conditions regardless of the presence or absence of Mg^{2+} and a substrate (Fig. 6D). The interaction between these two components might be attributable to their hydrophobic nature, since they are dissociated by SDS under non-reductive conditions. Our preliminary experiments showed that the addition of 0.3% Triton X-100 resulted in approximately 2-fold elevation of the enzymatic activity of the native HepPP synthase purified from *B. stearothermophilus* cells. However, the native HepPP synthase purified from *B. subtilis* (*14*) or the native HexPP synthase purified from *M. luteus* B-P 26 (*32*) was not activated by this detergent. This unique profile has been proposed to explain the enzymatic reaction mechanisms of HepPP synthase of *B. subtilis* and HexPP synthase of *M. luteus* B-P 26, by assuming their ability to allow efficient turnover of synthesis of water-insoluble products from soluble substrates without the aid of detergent-like molecules (*12, 30*). The exceptional properties of *B. stearothermophilus* HepPP synthase might be due to the hydrophobic interaction. As a result, this enzyme might obtain a thermostable nature.

On the other hand, the hybrid-type enzyme was found to be dissociated into the two subunits during gel filtration regardless of the presence or absence of Mg^{2+} and a substrate, whereas the formation of a small amount of the complex of component I-sub and component II-sub was observed, at a position corresponding to 60 kDa, in the presence of Mg^{2+} and a substrate (Fig. 6B). This fact indicates that the interaction between component I-sub and component II′-ste is so weak that the complex dissociates during gel filtration, resulting in predominant elution at around 30 kDa.

We are interested in the reason why only medium-chain prenyltransferases are heterodimers whereas all other three types of prenyltransferases are tightly coupled homodimers. This finding of the interchangeability of the hetero subunits will help us to study the roles of the components of these enzymes.

REFERENCES

1. Clark, S. (1992) Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu. Rev. Biochem.* **61**, 355-386

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

2. Ogura, K. and Koyama, T. (1997) Mechanistic enzymology and molecular genetics of chain elongation in isoprenoid biosynthesis in *Dynamic Aspects of Natural Products Chemistry* (Ogura, K. and Sankawa, U., eds.) pp. 1-23, Kodansha, Tokyo
3. Koike-Takeshita, A., Koyama, T., Obata, S., and Ogura, K. (1995) Molecular cloning and nucleotide sequences of the genes for two essential proteins constituting a novel enzyme system for heptaprenyl diphosphate synthesis. *J. Biol. Chem.* **270**, 18396-18400
4. Eberhardt, N.L. and Rilling, H.C. (1975) Prenyltransferase from *Saccharomyces cerevisiae*. Purification to homogeneity and molecular properties. *J. Biol. Chem.* **250**, 863-866
5. Sagami, H., Morita, Y., and Ogura, K. (1994) Purification and properties of geranylgeranyl diphosphate synthase from bovine brain. *J. Biol. Chem.* **269**, 20561-20566
6. Ishii, K., Sagami, H., and Ogura, K. (1986) A novel prenyltransferase from *Paracoccus denitrificans*. *Biochem. J.* **233**, 773-777
7. Muth, J.D. and Allen, C.M. (1984) Undecaprenyl pyrophosphate synthetase from *Lactobacillus plantarum*: A dimeric protein. *Arch. Biochem. Biophys.* **230**, 49-60
8. Adair, W.L., Jr., Cafmeyer, N., and Keller, R.K. (1984) Solubilization and characterization of the long chain prenyltransferase involved in dolichyl phosphate biosynthesis. *J. Biol. Chem.* **259**, 4441-4446
9. Ohnuma, S., Koyama, T., and Ogura, K. (1991) Purification of solanesyl-diphosphate synthase from *Micrococcus luteus*. A new class of prenyltransferase. *J. Biol. Chem.* **266**, 23706-23713
10. Fujii, H., Koyama, T., and Ogura, K. (1982) Hexaprenyl pyrophosphate synthetase from *Micrococcus luteus* B-P 26. *J. Biol. Chem.* **257**, 14610-14612
11. Yoshida, I., Koyama, T., and Ogura, K. (1987) Dynamic interaction between components of hexaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26. *Biochemistry* **26**, 6840-6845
12. Yoshida, I., Koyama, T., and Ogura, K. (1989) Formation of a stable and catalytically active complex of the two essential components of hexaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26. *Biochem. Biophys. Res. Commun.* **160**, 448-452
13. Fujii, H., Koyama, T., and Ogura, K. (1983) Essential protein factors for polyprenyl pyrophosphate synthetases. *FEBS Lett.* **161**, 257-260
14. Takahashi, I., Ogura, K., and Seto, S. (1980) Heptaprenyl pyrophosphate synthetase from *Bacillus subtilis*. *J. Biol. Chem.* **255**, 4539-4543
15. Ashby, M.N. and Edwards, P.A. (1990) Elucidation of the deficiency in two yeast coenzyme Q mutants. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase. *J. Biol. Chem.* **265**, 13157-13164
16. Asai, K., Fujisaki, S., Nishimura, Y., Nishino, T., Okada, K., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1994) The identification of *Escherichia coli ispB* (*cel*) gene encoding the octaprenyl diphosphate synthase. *Biochem. Biophys. Res. Commun.* **202**, 340-345
17. Anderson, M.S., Yarger, J.G., Burck, C.L., and Poulter, C.D. (1989) Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**, 19176-19184
18. Clarke, C.F., Tanaka, R.D., Svenson, K., Wamsley, M., Fogelman, A.M., and Edwards, P.A. (1987) Molecular cloning and sequence of a cholesterol-repressible enzyme related to prenyltransferase in the isoprene biosynthetic pathway. *Mol. Cell. Biol.* **7**, 3138-3146
19. Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchida, M., Nishino, T., and Ogura, K. (1993) Thermostable farnesyl diphosphate synthase of *Bacillus stearothermophilus*: Molecular cloning, sequence determination, overproduction, and purification. *J. Biochem.* **113**, 355-363
20. Wilkin, D.J., Kutsunai, S.Y., and Edwards, P.A. (1990) Isolation and sequence of the human farnesyl pyrophosphate synthetase cDNA. Coordinate regulation of the mRNAs for farnesyl pyrophosphate synthetase, 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, and 3-hydroxy-3-methyl-glutaryl coenzyme A synthase by phorbol ester. *J. Biol. Chem.* **265**, 4607-4614
21. Carattoli, A., Romano, N., Ballario, P., Morelli, G., and Macino, G. (1991) The *Neurospora crassa* carotenoid biosynthetic gene (*albino* 3) reveals highly conserved regions among prenyltransferases. *J. Biol. Chem.* **266**, 5854-5859
22. Math, S.K., Hearst, J.E., and Poulter, C.D. (1992) The *crtE* gene in *Erwinia herbicola* encodes geranylgeranyl diphosphate synthase. *Proc. Natl. Acad. Sci. USA* **89**, 6761-6764
23. Ohnuma, S., Suzuki, M., and Nishino, T. (1994) Archaebacterial ether-linked lipid biosynthetic gene. Expression cloning, sequencing, and characterization of geranylgeranyl-diphosphate synthase. *J. Biol. Chem.* **269**, 14792-14797
24. Shimizu, N., Koyama, T., and Ogura, K. (1998) Molecular cloning, expression, and characterization of the genes encoding the two essential protein components of *Micrococcus luteus* B-P 26 hexaprenyl diphosphate synthase. *J. Bacteriol.* **180**, 1578-1581
25. Zhang, Y.-W., Koyama, T., and Ogura, K. (1997) Two cistrons of the *gerC* operon of *Bacillus subtilis* encode the two subunits of heptaprenyl diphosphate synthase. *J. Bacteriol.* **179**, 1417-1419
26. Davisson, V.J., Woodside, A.B., Neal, T.R., Stremler, K.E., Muehlbacher, M., and Poulter, C.D. (1986) Phosphorylation of isoprenoid alcohols. *J. Org. Chem.* **51**, 4768-4779
27. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
28. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
29. Koyama, T., Fujii, H., and Ogura, K. (1985) Enzymatic hydrolysis of polyprenyl pyrophosphates. *Methods Enzymol.* **110**, 153-155
30. Zhang, Y.-W., Koyama, T., Marecak, D.M., Prestwich, G.D., Maki, Y., and Ogura, K. (1998) Two subunits of heptaprenyl diphosphate synthase of *Bacillus subtilis* form a catalytically active complex. *Biochemistry* **37**, in press
31. Iwaya, T. (1995) Master's thesis. Tohoku University, Sendai, Japan
32. Yoshida, I., Koyama, T., and Ogura, K. (1989) Protection of hexaprenyl-diphosphate synthase of *Micrococcus luteus* B-P 26 against inactivation by sulphydryl reagents and arginine-specific reagents. *Biochim. Biophys. Acta* **995**, 138-143

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012