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THE IDENTIFICATION OF *ESCHERICHIA COLI ispB* (*cel*) GENE ENCODING THE OCTAPRENYL DIPHOSPHATE SYNTHASE

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The gene located upstream of the *nlp* gene at min 69 on the chromosome of *Escherichia coli* was cloned from the plasmid pLC7-42 constructed by Clarke and Carbon. The extract of the strain harboring the plasmid containing this gene showed increased activity of the prenyltransferase. The product of the enzyme reaction was analyzed by two systems of the thin-layer chromatography and shown to be the phosphate ester of all-*E*-octaprenol. These results suggested that the gene was the structural gene for the octaprenyl diphosphate synthase which supplied the precursor of the side chain of the isoprenoid quinones.

The enzyme for isoprenoid biosynthesis, which catalyzes the condensation between an allyl diphosphate and isopentenyl diphosphate (IPP), is called a prenyltransferase. Although the composition of isoprenoids in the bacteria is simpler than that in the eukaryote, each species of bacteria such as *Bacillus subtilis* (1-3) and *Micrococcus luteus* (4, 5) have several kinds of prenyltransferases which generate the various prenyl diphosphates. Three prenyltransferases, namely, farnesyl diphosphate synthase (EC 2.5.1.10), octaprenyl diphosphate synthase, and undecaprenyl diphosphate synthase (EC 2.5.1.31) are found also in the extract of *Escherichia coli* (6).

The *ispA* gene encoding farnesyl diphosphate synthase is the only known gene for the prenyltransferase of *E. coli* (7). Recently, we determined the sequence of the *nlp* gene in min 69 in *E. coli* chromosome and found that an open-reading frame upstream of the *nlp* gene showed the homology with the *ispA* gene (8). Jeong *et al.* (9) also determined the sequence in this region and

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Abbreviations: IPP, isopentenyl diphosphate; FPP, all-*E*-farnesyl diphosphate.

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found that this open-reading frame encoded a protein homologous to the hexaprenyl diphosphate synthase of *Saccharomyces cerevisiae* (10) and the geranylgeranyl diphosphate synthase of various organisms (11, 12). They tentatively named this gene as *cel* (*crtE* - like gene). To determine the function of this gene, we cloned this gene from the plasmid pLC7-42 bearing this region (13) and measured the prenyltransferase activity of the strain harboring the plasmid containing this gene. The result of separation of the enzyme and the analysis of the reaction product showed the increased activity of octaprenyl diphosphate synthase. Thus, we concluded that this gene is the structural gene for octaprenyl diphosphate synthase and named the gene *ispB* (second gene for isoprenoid biosynthesis in *E. coli*).

MATERIALS AND METHODS

Materials — Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo and Toyobo Co. [1-^{14}C]IPP (1.96 TBq/mol) were from Amersham Co. IPP, dimethylallyl diphosphate, and all-*E*-farnesyl diphosphate (FPP) were synthesized by phosphorylation of the corresponding prenols (14). Solanesol (all-*E*-nonaprenol) was provided by Nisshin Flour Milling Co. Prenols (C_{40}-C_{60}) from *Ailanthus altissima* were purchased from Sigma Chemical Co.

Strains and plasmids — *E. coli* strain JM109 was used for the cloning of the *ispB* locus. Strain JA200 harboring plasmid pLC7-42 (15) was provided by Dr. A. Nishimura. Plasmids pCL1920, pCL1921 (16), and pUC119 (17) were used as the vectors. pCL1920 and pCL1921 were provided Dr. C. G. Lerner. Plasmid pKA3 was constructed from the 3 kb *Kpn* I - *Hin* dIII fragment of pLC7-42 inserted between the *Kpn* I and *Hin* dIII site of pCL1920 (Fig. 1). pKA3E was constructed by the insertion of *Eco* RI linker into the cleaved and blunted *Hin* dIII site of pKA3. pKA5 was constructed from the 3 kb *Kpn* I - *Eco* RI fragment of pKA3E inserted between the *Kpn* I and *Eco* RI site of pCL1921. pKA5 Δ 1 - 5 were constructed by the exonuclease III deletion (18) from the *Nru* I site of the pKA5. pKA7 was constructed from the 2.5 kb *Hin* dIII - *Eco* RI fragment of pKA5 Δ 3 inserted between the *Hin* dIII and *Eco* RI site of pUC119. The LB and LB agar media (19) were used for the growth of bacteria.

Preparation of the enzyme — Crude extracts were prepared from the *E. coli* cells by disruption in buffer containing 100 mM potassium phosphate (pH 7.4), 1 mM 2-mercaptoethanol, and 5 mM EDTA with a ultrasonic disintegrator six times for 30 s at 30-s interval. Cell extract was dialyzed against 10 mM potassium phosphate buffer (pH 7.5). The resulting solution was applied to Productiv DE column (Funakoshi) equilibrated with the same buffer. Elution was carried out with 20 mL of 120 mM NaCl in 10 mM potassium phosphate buffer (pH 7.5) (low-salt fraction), and then 20 mL of 300 mM NaCl in 10 mM potassium phosphate buffer (pH 7.5) (high-salt fraction). These fractions were used for enzyme reaction.

Prenyltransferase reaction — The incubation mixture contained, in a final volume of 0.1 mL, 0.1 μ mol of $MgCl_2$, 0.1 mg of Triton X-100, 5.0 μ mol of potassium phosphate buffer (pH 7.5), 1.0 nmol of [^{14}C] IPP (specific activity 0.92 TBq/mol), 0.5 nmol of FPP, and the enzyme. After incubation for 10 or 30 min at 30°C, the reaction was stopped by heating for 5 min at 90°C. The products were extracted with 1-butanol, and the radioactivity in the extracts was measured. The enzyme activity was expressed in terms of radioactivity in the 1-butanol extract.

Product analysis — Prenyl diphosphate was extracted with 1-butanol from the reaction mixture and hydrolysed with phosphatase in the way of Fujii *et al.* (20). The products of hydrolysis were extracted with hexane and analyzed by both normal phase and reversed phase thin-layer chromatography with reference prenols. The normal phase thin-layer chromatography was carried out on Kieselgel 60 F_{254} plate (Merck) with benzene-ethyl acetate (4:1, v/v). The reversed phase thin-layer chromatography was carried out on LKC18 plate (Whatman) with acetone -water (19:1, v/v). Radioactivity on the thin-layer chromatography plates was detected with Imaging analyzer BAS1000-Mac (Fuji film Co.) and the value of radioactivity was given in PSL (photo-stimulated luminescence, arbitrary unit). The spots of the marker prenols were visualized by exposure of the plate to iodine vapor.

RESULTS AND DISCUSSION

Cloning of *ispB* gene —— The plasmid pLC7-42 bears the region of min 69 on the chromosome of *E. coli* (13). The *Kpn* I - *Hin* dIII fragment containing the open-reading frame upstream of *nlp* (9) could be cloned into the low-copy number vector, pCL1920, while the attempt to clone the fragment into a high-copy number vector as pUC118/119 was unsuccessful (Fig. 1). We constructed the serial deletions from the resulting plasmid pKA5, and measured the activities of prenyltransferase in the extracts of the strains harboring the plasmids using [^{14}C]IPP and FPP as substrates (Table 1). The strain with the plasmid pKA5 Δ 3 which had a deletion of 0.60 kb from *Kpn* I site showed the increased prenyltransferase activity, but the strain with the plasmid pKA5 Δ 4 which had a deletion of 0.72 kb from *Kpn* I site showed the same activity as the strain with the vector alone. These results suggest that the open-reading frame coding for a polypeptide with a molecular weight of 35,215 described by Jeong *et al.* (9) is the gene for prenyltransferase. We name this gene *ispB* because this is the second prenyltransferase gene found in *E. coli.*

The growth of the strains harboring the plasmid pKA3 or pKA5 was slower than that of the strain harboring the vector alone, while the strain harboring the plasmid pKA5 Δ 1, pKA5 Δ 2, pKA5 Δ 3, pKA5 Δ 4, or pKA5 Δ 5 grew as fast as the strain harboring the vector alone (not shown). These results suggest that the increase in the dosage of the *rplU* gene which encodes ribosomal protein L21 inhibits the growth of the host strain. Then the attempt to clone the insert of the pKA5 Δ 3 into the pUC119 was made successfully, and the resulting plasmid pKA7 was used in the following experiment.

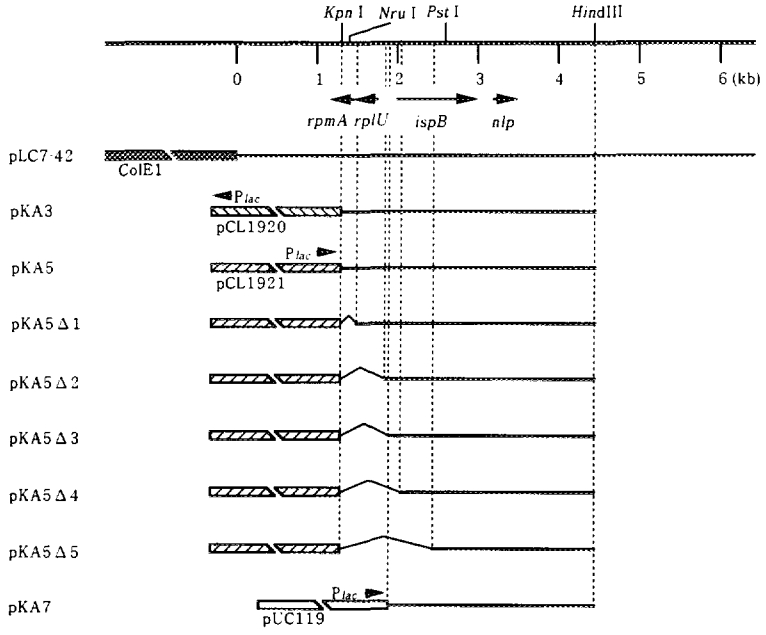

Fig. 1. Restriction map and the construction of the plasmids. The open-reading frames deduced from the nucleotide sequence by Jeong *et al.* (9) are indicated by the arrows. The plasmids in this figure except for pLC7-42 were constructed as described in MATERIALS AND METHODS.

Table 1. Prenyltransferase activity in the extract of the strains harboring various plasmids

Plasmid	Activity (dpm $\times 10^{-3}$)	
	−IPTG	+IPTG
pCL1920	1.3	0.9
pKA5Δ1	8.8	10.5
pKA5Δ2	13.7	11.6
pKA5Δ3	7.7	11.6
pKA5Δ4	1.1	0.8
pKA5Δ5	1.3	1.1

The strains were grown in 5 mL of LB media at 37°C to the early stationary phase with or without 1 mM of isopropyl thiogalactopyranoside (IPTG). The extract of the cells were prepared and the prenyltransferase activities were measured as described in MATERIALS AND METHODS. The cell extract added to a reaction mixture was 50 μg of protein and the incubation was carried out for 10 min. The enzyme activity was expressed as the radioactivity in the 1-butanol extract.

The function of the *ispB* product — The preparation and the fractionation of the extract from the strain harboring either pKA7 or no plasmid were carried out, and the prenyltransferase activities in each fraction were measured (Table 2). The specific activity of the prenyltransferase in high-salt fraction of the strain harboring the pKA7 was about ten times of that of the strain JM109. However, the prenyltransferase activities in the low-salt fraction of these strains were not markedly different from each other. The enzyme in the high-salt fraction seems to be the octaprenyl diphosphate synthase, because the octaprenyl diphosphate synthase is known to elute later than the undecaprenyl diphosphate synthase from the DEAE-Toyopearl column (6). To confirm this identification, the product generated in the reaction with high-salt fraction of the strain JM109/pKA7 was hydrolyzed by the phosphatase and analyzed by thin-layer chromatography. The alcohol derived from the product moved slightly more slowly than solanesol in normal phase

Table 2. Prenyltransferase activity in the fraction of the anion-exchange chromatography

Strain	Fraction	Conc. of protein (μg/mL)	Activity (pmol/min)	Specific activity (pmol/min/mg protein)
JM109	Low salt	142	0.25	35
	High salt	296	0.48	32
JM109/pKA7	Low salt	158	0.43	55
	High salt	258	4.39	340

The strains were grown in 100 mL of LB media for 18 h at 37°C. The preparation of the enzyme and the measurement of the enzyme activity was carried out as described in MATERIALS AND METHODS. To a reaction mixture was added 50 μL of the fraction, and the incubation was carried out for 30 min. The enzyme activity was expressed as picomoles of IPP incorporated into the products in the 1-butanol extract per milligram of protein per minute.

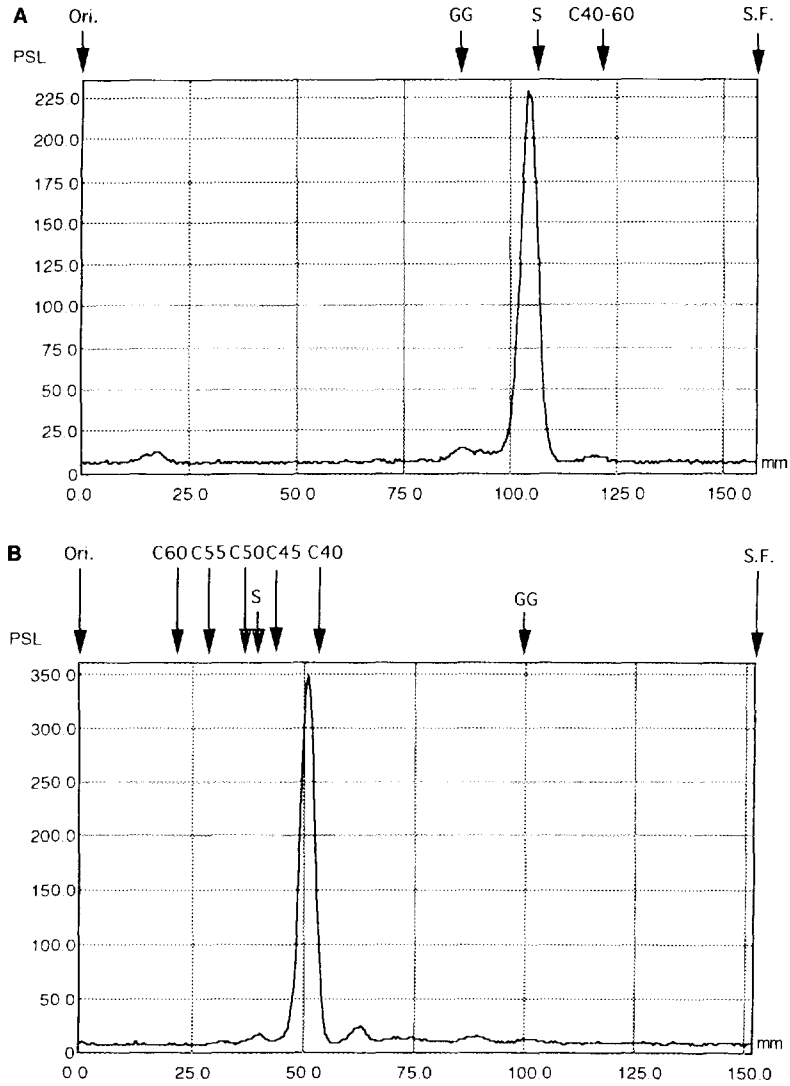

Fig. 2. Thin-layer chromatogram of the product in the enzyme reaction. The enzyme reaction with a high-salt fraction of JM109/pKA7 where [^{14}C]IPP and FPP were used as substrates was carried out. The product was hydrolyzed with the phosphatase (20), and the analysis of the resulting alcohol was carried out by normal phase thin-layer chromatography (A) and by reversed phase thin-layer chromatography (B) as described in MATERIALS AND METHODS.
Ori., origin; S.F., solvent front; GG, all-*E*-geranylgeraniol; S, solanesol; C40, *Z, E*-octaprenol; C45, *Z, E*-nonaprenol; C50, *Z, E*-decaprenol; C55, *Z, E*-undecaprenol; C60, *Z, E*-dodecaprenol.

thin-layer chromatography (Fig. 2A). In reversed phase thin-layer chromatography, it moved faster than solanesol and slightly more slowly than *Z,E*-octaprenol (Fig. 2B). These results indicate that the alcohol is all-*E*-octaprenol, and confirm that the enzyme in high-salt fraction is octaprenyl diphosphate synthase. Thus, the *ispB* gene is suggested to be the structural gene for octaprenyl diphosphate synthase.

Octaprenyl diphosphate is the precursor for the side chain of ubiquinone (21) and menaquinone (22) in *E. coli*. Because the chain length of the side chain of isoprenoid quinones are supposed to be determined by the prenyl diphosphate available for the condensation with the aromatic precursor (23, 24), it may be possible to alter the chain length of isoprenoid quinones in *E. coli* by controling the expression of cloned gene for prenyltransferase such as *ispB*, *COQ1* encoding hexaprenyl diphosphate synthase in yeast (10), or *crtE* encoding geranylgeranyl diphosphate synthase (11, 12). The over expression of the *ispB* and the other related genes will also give information about the rate-limiting step in the biosynthesis of the isoprenoid quinones. These experiments are now in progress.

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