J. Biochem. **137**, 395–400 (2005)
DOI: 10.1093/jb/mvi049

Disruption of the Structural Gene for Farnesyl Diphosphate Synthase in *Escherichia coli*

Shingo Fujisaki[1,*], Isao Takahashi[1], Hiroshi Hara[2,3], Kensuke Horiuchi[2], Tokuzo Nishino[4] and Yukinobu Nishimura[1]

[1]Department of Biomolecular Science, Faculty of Science, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510; [2]National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540; [3]Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, Saitama 338-8570; and [4]Department of Bioengineering, Faculty of Engineering, Tohoku University, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579

Received September 22, 2004; accepted January 8, 2005

The chromosomal *ispA* gene encoding farnesyl diphosphate synthase of *Escherichia coli* was disrupted by inserting a *neo* gene cassette. The null *ispA* mutants were viable. The growth yield of the mutants was 70% to 80% of that of the wild-type strain under aerobic conditions, and was almost the same as the wild-type under anaerobic conditions. The levels of ubiquinone-8 and menaquinone-8 were both significantly lower (less than 13% and 18% of normal, respectively) in the mutants than in the wild-type. The undecaprenyl phosphate level in the mutants was modestly lower (40% to 70% of normal) than in the wild-type strain. Thus the synthesis of all-*E*-octaprenyl diphosphate, the precursor of ubiquinone-8 and menaquinone-8, was decreased more severely than that of *Z,E*-mixed undecaprenyl diphosphate, the precursor of undecaprenyl monophosphates, under the conditions where the synthesis of farnesyl diphosphate was decreased. The condensation of isopentenyl diphosphate with dimethylallyl diphosphate was detected in the cell-free extracts of the mutants, although it was 5% of that in the wild-type strain. A low level of farnesyl diphosphate seems to be synthesized in the mutants by other prenyltransferases such as octaprenyl diphosphate synthase or undecaprenyl diphosphate synthase.

Key words: isoprenoid, farnesyl diphosphate, menaquinone, ubiquinone, undecaprenyl phosphate.

Abbreviations: FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate.

Isoprenoids are natural compounds synthesized from isopentenyl diphosphate (IPP), which is a five-carbon compound, *via* allylic diphosphates with various carbon numbers ($5 \times n$). Organisms have an appropriate set of prenyl diphosphate synthases that catalyze the condensation of IPP with allylic diphosphates to produce other allylic diphosphates with higher carbon numbers (*1*). Three genes for prenyl diphosphate synthases exist in the chromosome of *Escherichia coli*: *ispA* for farnesyl diphosphate (FPP) synthase (*2*, *3*), *ispB* for octaprenyl diphosphate synthase (*4*), and *rth* for undecaprenyl diphosphate synthase (*5*, *6*). FPP synthase catalyzes the sequential condensation of IPP with dimethylallyl diphosphate (C5) and geranyl diphosphate (C10) to form all-*E*-FPP (C15). Octaprenyl diphosphate synthase and undecaprenyl diphosphate synthase catalyze the sequential condensation of IPP with all-*E*-FPP to form all-*E*-octaprenyl diphosphate (C40) and a mixture of *Z,E*-mixed polyprenyl diphosphates (decaprenyl diphosphate (C50), undecaprenyl diphosphate (C55), and dodecaprenyl diphosphate (C60)), respectively (*1*, *7*). Octaprenyl diphosphate is a precursor of ubiquinone-8 and menaquinone-8 (*8*), and *Z,E*-mixed polyprenyl diphosphates are precursors of the *Z,E*-mixed polyprenyl monophosphates known as sugar carrier lipids (*9*). Both *ispB* (*10*) and *rth* (*6*) are known to be essential for growth. A lack of synthesis of either octaprenyl diphosphate or *Z,E*-mixed polyprenyl diphosphates is supposed to cause a shortage of indispensable compounds as isoprenoid quinones or sugar carrier lipids.

We have previously shown that a mutant of *E. coli* with a temperature-sensitive FPP synthase grew as well as the wild-type strain at 42°C, although the levels of isoprenoid quinones in the mutant were slightly lower than those in the wild-type strain (*2*). However, because there might have been residual temperature-sensitive FPP synthase activity, it was unclear whether FPP synthase is indispensable in *E. coli* or not. In the present study, we constructed null mutants of the *ispA* gene and showed that this gene is not essential for viability. We also characterized the growth properties and isoprenoid biosynthesis of the mutants.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—The *E. coli* K-12 strains used are listed in Table 1. SF6 and SF7 are the *ispA* disruptants characterized in this study, and SF5 is their isogenic control strain. Plasmid pHSG415s was used as a vector with a temperature-sensitive replication origin (*11*), and pHR12 (*12*) was used as the source of a

*To whom correspondence should be addressed. Phone: +81-47-472-1158, Fax: +81-47-475-1855, E-mail: sfujisak@biomol.sci.toho-u.ac.jp

Table 1. **Bacterial strains.**

Strain	Relevant characteristics or derivation	Source
W3110	wild type	Laboratory stock
FS1576	*recD1009 thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	(*18*)
MCL30	Δ (*srl-recA*) *306*::Tn*10*	(*19*)
GP407	*ispA*^{ts} Δ (*srl-recA*) *306*::Tn*10*	(*2*)
SF1	*ispA::neo* derivative of FS1576 by allelic exchange	This work
SF2	Δ*ispA::neo* derivative of FS1576 by allelic exchange	This work
SF3	*ispA::neo* derivative of W3110 by transduction (donor SF1)	This work
SF4	Δ*ispA::neo* derivative of W3110 by transduction (donor SF2)	This work
SF5	Δ (*srl-recA*) *306*::Tn*10* derivative of W3110 by transduction (donor MCL30)	This work
SF6	Δ (*srl-recA*) *306*::Tn*10* derivative of SF3 by transduction (donor MCL30)	This work
SF7	Δ (*srl-recA*) *306*::Tn*10* derivative of SF4 by transduction (donor MCL30)	This work

neo-containing kanamycin resistance cassette. Other plasmids used in this work are shown in Fig. 1. Plasmids pHX1 and pR60 were used as sources of the *ispA* gene (*3*). pSF14 was constructed by inserting the *neo*-containing 1.4-kb *Bam*HI fragment of pHR12 into the *Eco*T14I site of pHX1 after blunt-ending both fragments. pSF15 was constructed in the same way except that the pHX1 fragment was digested with exonuclease III and mung bean nuclease prior to blunt-end formation. The digestion deleted about 0.3 kb at each end of the fragment. The direction of the open reading frame of the *neo* was opposite to that of *ispA* in pSF14, but was same as that of *ispA* in pSF15. pSF27 was constructed by inserting the 1.3-kb *Pvu*II fragment of pR60 that carried *ispA*$^{+}$ under the *lac* promoter into the 5.6-kb *Sma*I–*Hin*cII fragment of the pHSG415s vector.

Chemicals—[1-^{14}C]IPP (specific activity, 2.04 TBq/mol) was purchased from Amersham Co. Unlabeled IPP, dimethylallyl diphosphate, and all-*E*-FPP were synthesized by phosphorylation of the corresponding prenols (*13*). Ubiquinone-10 and dodecaprenyl phosphate were purchased from Sigma Chemical Co.

Genetic and Recombinant DNA Procedures—The genetic and recombinant DNA procedures used were essentially based on the methods of Miller (*14*) and Sambrook *et al.* (*15*). P1*kc* was used for transduction (*14*). Southern blotting and PCR were carried out essentially as described previously (*15*). The 1.0-kb *Eco*RI–*Sal*I fragment of pR60 containing the *ispA* gene was labeled with [α-^{32}P]dCTP with a Random primer DNA labeling kit (Takara Co.) and then used as a probe. Forward primer 5′-GCGTACAAAT-TCTGCTGTCTG-3′ and reverse primer 5′-TCCCAAAT-CAATTGGTCAAACG-3′ were used to amplify the DNA fragment in the region including the open reading frame of *ispA* in PCR.

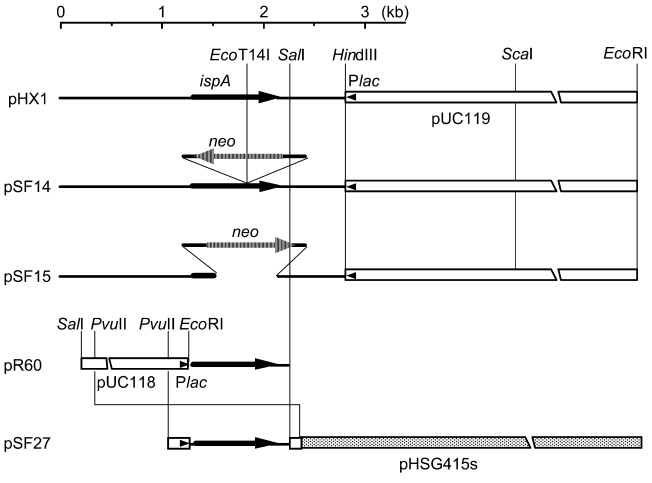

Fig. 1. **Plasmids used in this study and restriction maps of the plasmids.** Open boxes, small arrowheads, and the shaded box denote the vector regions of pUC118/119, the *lac* promoter in the vectors pUC118/119, and the vector region of pHSG415s, respectively. Solid arrows denote the coding regions of the *ispA* gene, and striped arrows denote those of the *neo* gene.

Growth of Cells—The cells used for the assaying of enzyme activities and the analysis of isoprenoids were grown in L-broth (*16*). Growth tests were carried out in Davis minimal medium supplemented with 0.2% glucose (*17*), or in double-strength Davis minimal medium supplemented with the indicated amount of glucose and 0.1% of casamino acids. The turbidity of cultures was measured hourly with a Fuji Digital Turbidometer at the wavelength of 660 nm.

IPP Isomerase and Prenyltransferase Activities—Enzyme activities were measured in a cell-free homogenate as described previously (*7*) with a slight modification. Cells from 50 ml of culture were disrupted in 1 ml of 100 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20% ethylene glycol with a Tomy UD-200 ultrasonic disintegrator (six times for 30 s at 30-s intervals). After removal of the debris fraction by centrifugation, the supernatant fraction was used as the cell-free homogenate. The reaction mixture for IPP isomerase contained, in a final volume of 0.1 ml, 1 μmol of $MgCl_2$, 5 μmol of potassium phosphate buffer (pH 7.5), 1.0 nmol of [1-^{14}C]IPP (5.5×10^4 dpm; specific activity, 0.92 TBq/mol), and the cell-free homogenate (50 μg of protein). After incubation for 2 h at 30°C, 20 μl of 6 M HCl was added to stop the enzyme reaction and to hydrolyze the reaction product, and then the mixture was incubated for 20 min at 37°C. The products of acid treatment were extracted with diethylether, and the radioactivity in the extract was measured after washing the extract with a saturated NaCl solution. The reaction mixture for the condensation of IPP with dimethylallyl diphosphate contained, in a final volume of 0.1 ml, 0.5 μmol of $MgCl_2$, 0.01 μmol of $MnCl_2$, 1 μmol of 2-mercaptoethanol, 5 μmol of potassium phosphate buffer (pH 7.5), 1.0 nmol of [1-^{14}C]IPP, 5 nmol of dimethylallyl diphosphate, and the cell-free homogenate (50 μg of protein). After incubation for 30 min at 30°C, 20 μl of 6 M HCl was added to stop the

enzyme reaction and to hydrolyze the reaction product, and then the mixture was incubated for 20 min at 37°C. The products of acid treatment were extracted with ligroin, and the radioactivity in the extract was measured after washing it with water. The radioactivity was corrected by subtracting from the observed value the background radioactivity (170 dpm), which was determined for an extract obtained from an incubation mixture to which HCl was added immediately after adding the substrates. The reaction mixture for the condensation of IPP with FPP contained, in a final volume of 0.1 ml, 0.1 μmol of $MgCl_2$, 0.1 mg of Triton X-100, 5 μmol of potassium phosphate buffer (pH 7.5), 1.0 nmol of [1-^{14}C]IPP, 0.5 nmol of FPP, and the cell-free homogenate (50 μg of protein). After incubation for 30 min at 30°C, the reaction was stopped by heating for 3 min at 95°C. The products were extracted with 1-butanol saturated with water.

Extraction and Analysis of Isoprenoids—Extraction and analysis of isoprenoids were carried out as described previously (*6*).

RESULTS

Isolation of ispA::neo Insertion Mutants—The construction of the disruptants involved the following steps, disruption of the *ispA* gene cloned in the pUC119 vector and replacement of the chromosomal *ispA* locus with the disrupted gene. First, the cassette containing the *neo* gene was inserted into the cloned *ispA* gene to construct plasmids pSF14 and pSF15. The genes in pSF14 and pSF15 were designated as *ispA*::*neo* and Δ*ispA*::*neo*, respectively, because the latter gene lacked about two-thirds of the coding region of the *ispA* gene. Then *E. coli recD* strain FS1576 (*18*), harboring plasmid pSF27 that contained *ispA*$^+$, was further transformed with a plasmid, pSF14 or pSF15, that had been linearized by digestion with *Sca*I. From among the kanamycin-resistant transformants, ampicillin-sensitive colonies that were devoid of a plasmid, pSF14 or pSF15, were selected, yielding insertion mutants SF1/pSF27 and SF2/pSF27. Transductants, SF3/pSF27 and SF4/pSF27, were obtained by introduction of the mutation in SF1/pSF27 or SF2/pSF27, respectively, into the chromosome of parental strain W3110/pSF27 by P1-mediated transduction. The *recA* gene was inactivated by P1 transduction with MCL30 (*19*) as a donor yielding mutants SF6/pSF27 and SF7/pSF27. Both SF6 and SF7 were found to be able to grow after elimination of plasmid pSF27 by cultivation at 42°C, showing that *ispA* is dispensable for viability. Moreover, the disrupted genes in SF6 and SF7 could be transduced directly into W3110 by means of P1 phages grown in SF6 and SF7.

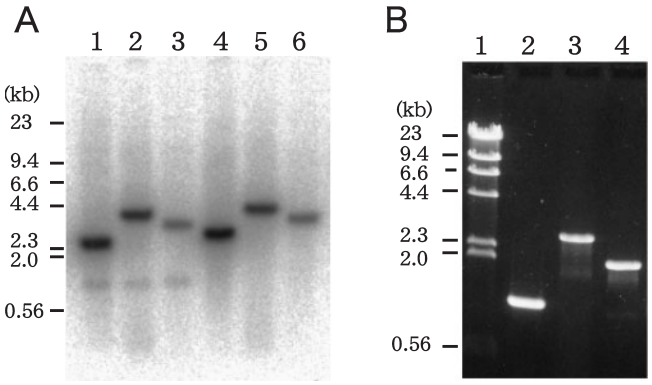

Fig. 2. **Southern blot and PCR analyses of the chromosomes of the *ispA* disruptants and the wild-type strain.** (A) Chromosomal DNA preparations from SF5 (*ispA*$^+$), SF6 (*ispA*::*neo*), and SF7 (Δ*ispA*::*neo*) were digested with *Bgl*I or *Eco*RV at 37°C overnight. The resulting fragments were separated by electrophoresis in a 0.7% agarose gel, transferred to a positively charged nylon membrane filter, and then probed with ^{32}P-labeled 1.0-kb *Eco*RI–*Sal*I fragments containing the *ispA* gene. Lanes: 1, SF5/*Bgl*I; 2, SF6/*Bgl*I; 3, SF7/*Bgl*I; 4, SF5/*Eco*RV; 5, SF6/*Eco*RV; 6, SF7/*Eco*RV. (B) DNA fragments of the *ispA* region were amplified from chromosomal preparations of SF5, SF6, and SF7 on PCR. The resulting fragments were separated by electrophoresis in a 0.85% agarose gel. Lanes: 1, molecular weight markers (λ/*Hin*dIII); 2, SF5; 3, SF6; 4, SF7.

The presence of a single insertionally inactivated *ispA* gene in the chromosome was confirmed by Southern blot analysis of the strains (Fig. 2A). It revealed 1.5 and 2.7-kb fragments in *Bgl*I digests of chromosomal DNA obtained from SF5. The 1.5-kb fragment was also found in *Bgl*I digests of chromosomal DNA obtained from SF6 and SF7. It revealed 4.1 and 3.5-kb fragments that should contain the 1.4-kb *neo*-fragment in *Bgl*I digests of chromosomal DNA obtained from SF6 and SF7. Fragments of 3.0, 4.4, and 3.8 kb were found in *Eco*RV digests from SF5, SF6, and SF7, respectively. Moreover, 1.2, 2.6, and 2.0-kb fragments were amplified on PCR from the chromosomal DNA preparations from SF5, SF6, and SF7, respectively (Fig. 2B). Thus, all the results indicated that there were insertion of the 1.4-kb *neo* fragment into the *ispA* genes of SF6 and SF7, and the deletion of a 0.6-kb fragment in the *ispA* gene of SF7.

Growth Properties of the ispA::neo Insertion Mutants—*ispA* mutants SF6 and SF7 could grow in the minimal medium supplemented with glucose, glycerol, or succi-

Table 2. **Growth yields under aerobic and anaerobic conditions.**[a]

Strain	Growth yield at A_{660}[b]			
	Aerobic[c]		Anaerobic[d]	
Glucose conc.	2 mM	4 mM	2 mM	4 mM
SF5	0.689 (0.090)	1.043 (0.092)	0.233 (0.028)	0.382 (0.037)
SF6	0.477 (0.060)	0.736 (0.071)	0.234 (0.006)	0.374 (0.022)
SF7	0.557 (0.083)	0.689 (0.176)	0.242 (0.021)	0.376 (0.018)

[a]Values shown represent averages of results obtained in three assays. The numbers in parentheses indicate standard deviations. [b]Turbidity was measured after 12 h cultivation. [c]Cells were grown at 37°C in 20 ml of the medium in a 100 ml flask with shaking at 100 rpm. [d]Cells were grown at 37°C in 11 ml of the medium in a capped tube in an anaerobic pouch.

Table 3. **Isoprenoid levels in strains SF5, SF6, and SF7.**[a]

Strain	Level (mg/g [dry wt] of cells)[b]					
	UQ-8	MK-8	C_{50}-P	C_{55}-P	C_{60}-P	Total Pol-P
SF5	529 (57)	232 (50)	66 (10)	360 (80)	41 (33)	467
SF6	26 (7)	31 (8)	11 (8)	150 (23)	26 (6)	187
SF7	67 (14)	41 (11)	12 (0)	253 (28)	59 (6)	324

[a]Values shown represent averages of results obtained in at least three assays. The numbers in parentheses indicate standard deviations. [b]Abbreviations: UQ-8, ubiquinone-8; MK-8, menaquinone-8; C_{50}-P, decaprenyl phosphate; C_{55}-P, undecaprenyl phosphate; C_{60}-P, dodecaprenyl phosphate; Pol-P, polyprenyl phosphates.

nate as a carbon source, although the growth rates were lower than those of the wild-type strain. The doubling times of SF6 and SF7 under aerobic conditions in the glucose minimal medium at 37°C were 137 ± 20 min and 106 ± 13 min, respectively, while that of wild-type control strain SF5 was 75 ± 3 min. The growth yields of the mutants in the glucose minimal medium were 70% to 80% of that of the wild-type strain under aerobic conditions, however, the growth yields of the mutants were almost as high as that of the wild-type strain under anaerobic conditions (Table 2). There was no detectable anomaly in the morphology of the mutant cells under either conditions.

Enzyme Activities in the ispA Mutants—The activity of the condensation of IPP with dimethylallyl diphosphate in the cell-free homogenate of temperature-sensitive *ispA* mutant GP407 was modestly lower than that of wild-type strain SF5 (Fig. 3). The decreases in the activities of *ispA* null mutants SF6 and SF7 were more severe, but there were detectable activities in the homogenates of the mutants even after subtracting the background value obtained for a zero-time control sample. The activities in the homogenates of both SF6 and SF7 were 5% of that of SF5. The rates of isomerization of IPP and condensation of IPP with FPP were almost the same in the cell-free homogenates of the mutants as those of the wild-type strain (not shown).

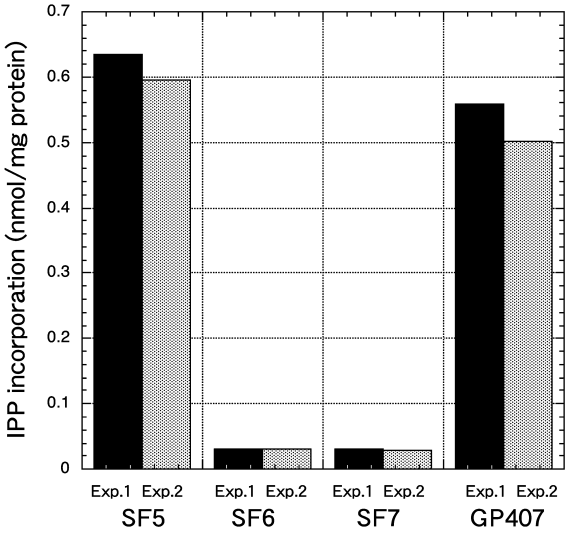

Fig. 3. **FPP synthase activities in homogenates of the *ispA* mutants and wild-type strain.** The activity of condensation of IPP with dimethylallyl diphosphate was measured as described under "MATERIALS AND METHODS." The values, expressed as nanomoles of IPP incorporated into the acid-labile products per milligram of protein during 30 min, obtained in two assays are shown.

Isoprenoid Contents of the ispA::neo Insertion Mutants—Isoprenoids were extracted from the cells in the early stationary phase and analyzed. The levels of isoprenoid quinones in the null mutants were much lower than those in the wild-type strain (Table 3). The ubiquinone levels in SF6 and SF7 were 5% and 13% of that in SF5, and the menaquinone levels in these mutants were 13% and 18% of that in SF5, respectively. On the other hand, the levels of *Z,E*-mixed polyprenyl phosphates in the null mutants were decreased modestly. The levels of total polyprenyl phosphates (the sum of the decaprenyl phosphate, undecaprenyl phosphate, and dodecaprenyl phosphate levels) in SF6 and SF7 were 40% and 69% of that in SF5.

DISCUSSION

The null *ispA* mutants were able to grow without the plasmid that carried the wild-type *ispA* gene, which showed that *ispA* was nonessential, at least in the genetic background of strain W3110. Actually, some activity of the condensation of IPP with dimethylallyl diphosphate occurred in the *ispA* null mutants, although it was only about 5% of that in the wild-type strain. This residual activity should be responsible for the synthesis of the decreased level of isoprenoids in the mutants. The decreases in the isoprenoid level and growth rate were more severe for SF6 than SF7, in spite of that they showed almost the same residual chain elongating activity with dimethylallyl diphosphate. This difference might be due to a polar effect of the inserted *neo* gene. There is an open reading frame of the *dxs* gene for deoxyxylulose synthase, the enzyme for the first reaction in the isoprenoid biosynthesis pathway, just downstream of the *ispA* gene in the chromosome of *E. coli* (*20*, *21*), and these genes seem to be transcribed in a single messenger RNA (*21*). As the direction of the *neo* gene in SF6 is opposite to that of *ispA* and *dxs*, the expression of the *dxs* gene is supposed to be decreased to cause a decrease in the synthesis of IPP in SF6. A shortage of IPP may cause a more severe decrease in isoprenoid biosynthesis.

The growth yields of the two mutants were as high as that of the wild-type strain under anaerobic conditions, although they were slightly lower than that of the wild-type strain under aerobic conditions. This phenotype seems to be caused by a decrease in the level of ubiquinone, because a similar but more severe growth defect has been reported for strains lacking ubiquinone (*8*). In fact, the levels of ubiquinone in the *ispA* null mutants were significantly lower than in the wild-type strain,

but a detectable level of ubiquinone in the mutants remained, unlike in the *ubiA* mutants that lacked 4-hydroxybenzoate polyprenyltransferase (*8*).

The decrease in the *Z,E*-mixed polyprenyl phosphate level was only modest in the mutants. This result is consistent with our earlier observation that the *Z,E*-mixed polyprenyl phosphate level in a temperature-sensitive *ispA* mutant was as high as that in the wild-type strain (*2*). The increased IPP caused by the decrease in FPP synthase activity is supposed to have been utilized for the synthesis of *Z,E*-mixed polyprenyl phosphates rather than for the synthesis of isoprenoid quinones *via* all-*E*-octaprenyl diphosphate. The concentration of IPP has been reported to have an important influence on the metabolic flow to various isoprenoids (*22*). Also, the rate of synthesis of *Z,E*-mixed polyprenyl phosphate seems to be controlled by the level of IPP. A decrease in the synthesis of IPP, due either to the specific inhibitor fosmidomycin (*23*) or a mutation in the gene for biosynthesis (*24*), causes cells to swell, which could be the direct consequence of the inhibition of cell wall synthesis by the decrease in *Z,E*-mixed polyprenyl phosphates, as has been shown for the cells of a temperature-sensitive mutant of the *rth* gene for undecaprenyl diphosphate synthase (*6*). The cell shape of the *ispA* mutant was normal, showing that the level of polyprenyl phosphates in the mutant (40% of that in the wild-type strain) was adequate for cell wall synthesis.

There should be some enzymes for the residual activity for condensation of IPP with dimethylallyl diphosphate in the mutants. However, it is unlikely that there is another gene for FPP synthase in the chromosome of *E. coli*, because *ispB*, which encodes octaprenyl diphosphate synthase, is the only gene that exhibits significant sequence homology with *ispA* (*4*, *25*). Either octaprenyl diphosphate synthase or undecaprenyl diphosphate synthase might be the enzyme responsible for the residual activity in the mutant cells, because both of these enzymes show slight activities as to the condensation of IPP with dimethylallyl diphosphate *in vitro* (*7*). The product specificities of medium-chain length polyprenyl diphosphate synthases of other organisms are known to change with the reaction conditions such as the concentrations of substrates and magnesium ion (*1*, *26*). Moreover, the enzyme protein forms complexes with other proteins (*27*), and the product specificity is known to change with complex formation (*28*). The product specificity of geranylgeranyl diphosphate synthase has also been reported to change with the formation of a complex with the small subunit of geranyl diphosphate synthase (*29*). Thus, it is possible that octaprenyl diphosphate synthase or undecaprenyl diphosphate synthase of *E. coli* can synthesize FPP from IPP and dimethylallyl diphosphate under appropriate conditions, and can substitute for FPP synthase in the cells of *ispA* null strains.

We wish to thank Dr. Akiko Nishimura, National Institute of Genetics, and Dr. Atsushi Higashitani, Tohoku University, for the valuable discussions, and for providing the strains and plasmids. Thanks are also due to Dr. Jun-ichi Kato, Tokyo Metropolitan University, for a helpful suggestion. This research was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Cooperated Research Program of National Institute of Genetics.

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