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Isolation and Characterization of an *Escherichia coli* Mutant Having Temperature-Sensitive Farnesyl Diphosphate Synthase

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The screening of a collection of highly mutagenized strains of *Escherichia coli* for defects in isoprenoid synthesis led to the isolation of a mutant that had temperature-sensitive farnesyl diphosphate synthase. The defective gene, named *ispA*, was mapped at about min 10 on the *E. coli* chromosome, and the gene order was shown to be *tsx-ispA-lon*. The mutant *ispA* gene was transferred to the *E. coli* strain with a defined genetic background by P1 transduction for investigation of its function. The in vitro activity of farnesyl diphosphate synthase of the mutant was 21% of that of the wild-type strain at 30°C and 5% of that at 40°C. At 42°C the ubiquinone level was lower (66% of normal) in the mutant than in the wild-type strain, whereas at 30°C the level in the mutant was almost equal to that in the wild-type strain. The polyprenyl phosphate level was slightly higher in the mutant than in the wild-type strain at 30°C and almost the same in both strains at 42°C. The mutant had no obvious phenotype regarding its growth properties.

It is well known that 3-hydroxy-3-methylglutaryl coenzyme A reductase is a rate-limiting enzyme in the biosyntheses of isoprenoid compounds, undergoing multivalent-feedback regulation (4). However, a branch point of the synthetic pathway of various isoprenoids seems to be a reaction utilizing isopentenyl diphosphate (IPP) or farnesyl diphosphate (FPP) or both. For elucidation of the mechanism by which the syntheses of various isoprenoids are regulated differently, many researchers have studied the regulation of activities of the enzymes, utilizing IPP or FPP or both as a substrate (1, 5–7, 11).

Escherichia coli includes isoprenoid quinones (ubiquinone-8, menaquinone-8, and demethylmenaquinone-8) having an all-*E*-octaprenyl side chain (8) and sugar carrier lipid (decaprenyl phosphate, undecaprenyl phosphate, and dodecaprenyl phosphate) containing a *Z,E*-mixed isoprenoid chain (27). Because the composition of isoprenoids in *E. coli* is simpler than that in eucaryotes, *E. coli* is a useful system as a model for studying the biosynthesis of nonsterol isoprenoids and its regulation. We have studied the biosynthetic enzymes, utilizing IPP as a substrate in *E. coli*, and separated IPP isomerase and three prenyltransferases, namely, FPP synthase, octaprenyl diphosphate synthase, and undecaprenyl diphosphate synthase, from each other (13). FPP synthase catalyzes the condensation of IPP with dimethylallyl diphosphate (C_5) or geranyl diphosphate (C_{10}) to give FPP (C_{15}). The latter two enzymes catalyze the sequential condensation of IPP with FPP to give long-chain polyprenyl diphosphates which are precursors of isoprenoid quinones and sugar carrier lipid.

One effective approach to the elucidation of the role of a certain enzyme in vivo is to isolate the mutant containing the defective enzyme and to characterize it. We screened a collection of temperature-sensitive mutants of *E. coli* (24) for defects in isoprenoid synthesis by measuring ^{14}C incorporation from [^{14}C]IPP into isoprenoids in the permeabilized cells (12). One of the mutants showed decreased ^{14}C incorporation and turned out to have temperature-sensitive FPP synthase. We mapped the mutant gene *ispA* at min 10 on the chromosome map of *E. coli* (2). Although the *ispA* mutant showed markedly decreased activity of FPP synthase at 40°C compared with the control *ispA*$^+$ strain, the isoprenoid level in the mutant grown at 42°C did not decrease so markedly except that the ubiquinone level in the mutant was 66% of that in the control. The mutant showed no obvious phenotype in growth properties.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. The *E. coli* K-12 strains used are listed in Table 1. GC4670 was provided by I. B. Holland, University of Leicester. P1 *kc* was used for transductions (19).

Media. L broth and L agar (18) were used. Thymine (40 mg/liter) and cytosine (40 mg/liter) were added, when required. Davis minimal medium (10) supplemented with 0.36% glucose and the other required compounds was used.

Chemicals. [1-^{14}C]IPP (53 Ci/mol) was purchased from Amersham Corp. Geraniol and *E,E*-farnesol were provided by Takasago Perfumary Co. Ficaprenols were provided by Nisshin Flour Milling Co. Dimethylallyl alcohol and solanesol were purchased from Tokyo Kasei Kogyo Co. and Sigma Chemical Co., respectively. Monophosphates and diphosphates of prenols were synthesized by phosphorylation of the corresponding prenols as described previously (17). Ubiquinone-8 and menaquinone-8 were isolated from *E. coli* W3110 as described previously (3).

Screening of temperature-sensitive mutants for defects in isoprenoid synthesis. Temperature-sensitive mutants were isolated from *E. coli* K-12 strain PA3092 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (24). They were grown in 7 ml of L-broth medium at 30°C to reach the stationary phase. After incubation for 20 min at 42°C, the cells were collected and lyophilized as described previously

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TABLE 1. Bacterial strains

Strain	Relevant characteristics or derivation	Source
PA3092	F⁻ *thr leu trp his thyA argH thi lacY malA mtl xyl tonA supE str*	GSRC[a]
JE11046	*ispA* temperature-sensitive mutant derived from PA3092 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, Gln⁻ Pyr⁻	This work
P4X8	Hfr *met*	GSRC
JE7858	F⁺ *zai*::Tn*10 proC tsx*::Tn*5 acrA purE*	GSRC
GC4670	*thr leu lacY lon*::Tn*10*	I. B. Holland
LC102	F⁻ *lacY proC tsx purE gal trp his arg str xyl mtl ile thi met ara leu*	GSRC
MV1184	Δ(*srl-recA*)*306*::Tn*10*	GSRC
GM306	*ispA* P1-sensitive derivative of JE11046 by cross with P4X8	This work
GP407	*ispA* Δ(*srl-recA*)*306*::Tn*10* derivative of GM306 by transduction (donor MV1184)	This work
GP701	*ispA lon*::Tn*10* derivative of GM306 by transduction (donor GC4670)	This work
GM2105	F⁻ *lacY proC tsx* Leu⁺ derivative of LC102 by cross with P4X8	This work
GP1009	*ispA*⁺ Pro⁺ derivative of GM2105 by transduction (donor GP701)	This work
GP1011	*ispA* Pro⁺ derivative of GM2105 by transduction (donor GP701)	This work

[a] GSRC, Genetic Stock Research Center of the National Institute of Genetics, Mishima, Japan.

(12). To the lyophilized cells was added 0.1 ml of 50 mM potassium phosphate buffer (pH 7.4) containing [^{14}C]IPP, and the mixture was incubated for 20 min at 42°C. The cells were collected, and ^{14}C incorporation into cellular isoprenoids was measured.

IPP isomerase and prenyltransferase activity. Separation of each enzyme and the measurement of the activities of the partially purified enzymes were as described previously, except that [^{14}C]IPP and geranyl diphosphate were used as a substrate in the assay of FPP synthase (13). FPP synthase activity in crude extracts was assayed as follows. Crude extracts were prepared from the *E. coli* cells by disruption in buffer containing 100 mM potassium phosphate (pH 7.4), 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20% ethylene glycol with a Tomy UR-150P ultrasonic disintegrator six times for 20 s at 20-s intervals. After removal of a debris fraction by centrifugation, the extracts were used for assay of enzyme activity. The reaction mixture contained 50 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 0.1 mM $MnCl_2$, 10 mM 2-mercaptoethanol, 45 μM dimethylallyl diphosphate, 9 μM [^{14}C]IPP, and enzyme (100 μg of protein) in a final volume of 0.2 ml. After incubation for 30 min at 30 or 40°C, 50 μl of 6 M HCl was added to stop the enzyme reaction. Hydrolysis and extraction of the reaction products were carried out as described previously (13).

Extraction of isoprenoids. Extraction of isoprenoid quinones was carried out as described by Minnikin et al. (20) with a slight modification. The lyophilized cells (approximately 20 mg [dry weight]) were suspended in 2 ml of methanol–0.3% aqueous NaCl (10:1, vol/vol) and disrupted by sonication. To the suspension were added solutions of ubiquinone-10, phylloquinone, and solanesyl phosphate as an internal standard for the quantitation of isoprenoids. The mixture was shaken with 2 ml of hexane for extraction of isoprenoid quinones. The upper hexane layer was collected, and the extraction was repeated two more times. Phosphate ester derivatives of polyprenols were extracted from the residual aqueous layer as described by Crick and Carroll (9). To the aqueous layer was added 1 ml of 60% KOH, and the mixture was heated in a boiling water bath for 60 min. After hydrolysis of the phosphate ester derivatives with alkali, hydrolysis products, polyprenyl monophosphates, were extracted with diethyl ether. The ether extract was washed with 5% acetic acid and dried under N_2.

Analysis of isoprenoids. The hexane extract described above was loaded on a 0.4-g column of neutral alumina (grade III). Menaquinone and demethylmenaquinone were eluted with 2% diethyl ether in hexane, and ubiquinone was eluted with 6% diethyl ether in hexane as described previously (3). Isoprenoid quinones were analyzed by high-performance liquid chromatography, using a Shim-pack CLC-ODS reversed-phase column (4.6 by 150 mm; eluent, 99.5% ethanol; flow rate, 1 ml/min; column temperature, 40°C). Isoprenoid quinones were monitored at 248 or 275 nm with a spectrophotometric detector. The ether extract was suspended in chloroform and loaded on a 0.1-g column of silicic acid. The column was washed with chloroform, and prenyl phosphates were eluted with chloroform-methanol (1:4, vol/vol). The eluate was dried under N_2 and taken up in 2-propanol–methanol (1:1 [vol/vol], containing 5 mM phosphoric acid). High-performance liquid chromatography was performed using the same column as described above. The eluent was 2-propanol–methanol (1:1 [vol/vol], containing 5 mM phosphoric acid), and the flow rate was 1 ml/min. The wavelength used for monitoring prenyl phosphates was 210 nm. The amount of each compound was calculated from the peak area ratio relative to the internal standard.

RESULTS

Isolation of an *E. coli* mutant defective in isoprenoid synthesis. There seems to be no easy selection method available for the isolation of mutants defective in isoprenoid synthesis since the final products and intermediates in isoprenoid biosynthesis are impermeable to *E. coli* cells. Thus, we screened the temperature-sensitive mutants randomly by analyzing the radioactive products from [^{14}C]IPP in permeabilized cells (12). Among the 150 temperature-sensitive mutants examined, one strain, JE11046, showed decreased ^{14}C incorporation into isoprenoids at 42°C.

To find the defective enzyme in the mutant, we performed a partial purification of IPP isomerase and three prenyltransferases of JE11046 and its parent strain, PA3092, and measured the activities of them at 30 and 40°C. FPP synthase activity of the mutant at 40°C was 14% of that at 30°C, and the activity of the parent strain at 40°C was 94% of that at 30°C. The three other enzymes showed no significant difference between the two strains (data not shown). This indicates that FPP synthase of the mutant is temperature sensitive compared with that of the parent strain. The addition of FPP synthase of the mutant to the enzyme of the parent strain was not inhibitory, indicating that the decrease in activity of the mutant enzyme was not due to an inhibitory component, if any. The gene locus involved in the defect of FPP synthase was named *ispA* (for isoprenoid synthesis).

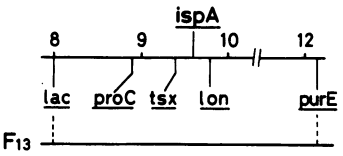

FIG. 1. Location of *ispA* on the *E. coli* chromosome with the reference markers.

Mapping of the *ispA* gene on the *E. coli* chromosome. Since JE11046 is derived from a heavily mutagenized culture of *E. coli*, the transfer of the mutant *ispA* gene to a defined background is necessary for analysis of the mutant phenotype. Therefore, the map position of *ispA* on the *E. coli* chromosome was determined. Since the phenotype of the *ispA* mutant is not easily distinguishable from that of the *ispA*$^+$ strain, the *ispA* mutation of JE11046 was mapped by the indirect mapping technique described previously (15). *ispA* mutant JE11046 was crossed with strain P4X8, and the selection was made on one or several markers carried by JE11046. Then the *ispA* Hfr recombinant was crossed with strain JE7858. Dozens of recombinants were analyzed for the unselected *ispA* marker by measurement of enzyme activity in vitro, and in this way *ispA* was found to be located between the *proC* and the *acrA* markers. The introduction of the F′ factor, F13 (16), as indicated in Fig. 1, into strain GP407 (*ispA recA*) converted the phenotype from IspA$^-$ to IspA$^+$. This result supports the idea that *ispA* is linked to the *proC-acrA* region and further demonstrates that the *ispA* mutation is recessive to the wild-type *ispA*$^+$ allele.

A derivative of *ispA* mutant GM306 was transduced by the P1 lysate of strain GC4670 (*lon*::Tn*10*), and the colonies resistant to tetracycline were selected. Among 10 *lon*::Tn*10* transductants examined for *ispA*, 7 were found to be converted to IspA$^+$ and 3 were IspA$^-$. Among the latter, GP701 (*ispA lon*::Tn*10*) was used as a donor for transducing strain GM2105 (*proC tsx lon*$^+$, a derivative of strain LC102). Selection of the recombinant was made on tetracycline resistance or the Pro$^+$ phenotype. Four *lon*::Tn*10 tsx*$^+$ transductants all proved to be IspA$^-$, and one of five *lon*::Tn*10 tsx* transductants was IspA$^-$, whereas the other four transductants were IspA$^+$. Two *proC*$^+$ *tsx*$^+$ *lon*::Tn*10* transductants were all IspA$^-$, and three *proC*$^+$ *tsx* transductants were all IspA$^+$. Of 15 *proC*$^+$ *tsx*$^+$ *lon*$^+$ transductants, 2 were IspA$^-$ and the other 13 were IspA$^+$. These results indicate that *ispA* was located in the *tsx-lon* region (Fig. 1).

The transductants, namely, GP1009 (*proC*$^+$ *tsx*$^+$ *ispA*$^+$) and GP1011 (*proC*$^+$ *tsx*$^+$ *ispA*), were used for more detailed characterization of the *ispA* mutation as described in the following sections.

Properties of the *ispA* mutant. The in vitro activity of FPP synthase of the mutant was significantly lower than that of the wild-type strain. The activities, expressed as nanomoles of IPP incorporated into the acid-labile products per milligram of protein during 30 min, in the extracts of GP1009 at 30 and 40°C were 1.64 ± 0.09 and 0.26 ± 0.04 (averages and standard deviations of the results from four assays), respectively, and those of GP1011 at 30 and 40°C were 0.34 ± 0.09 (21% of that of GP1009) and 0.013 ± 0.005 (5% of that of GP1009), respectively. There was no difference between them in growth properties. The growth rate and the final A_{660} of GP1011 in L-broth medium were almost equal to those of GP1009 at 30, 42, and 44°C. In L-broth medium deprived of NaCl or Davis minimal medium, there was no difference in growth between the two strains either.

To determine whether the decrease in activity of FPP synthase affects isoprenoid synthesis, it is necessary to determine the levels of isoprenoids in the cells. Analysis of the isoprenoids was carried out by high-performance liquid chromatography (Fig. 2). Each compound was identified by a comparison with the authentic compound. Peaks A (Fig. 2a) and C (Fig. 2b) were assigned to ubiquinone-8 and menaquinone-8, respectively. Peak B (Fig. 2b) was presumed to be demethylmenaquinone-8 from the retention time relative to menaquinone-8 (14). Peaks D, E, and F (Fig. 2c) eluted slightly earlier than the respective authentic ficaprenyl phosphates (mixture of C_{50}, C_{55}, and C_{60} com-

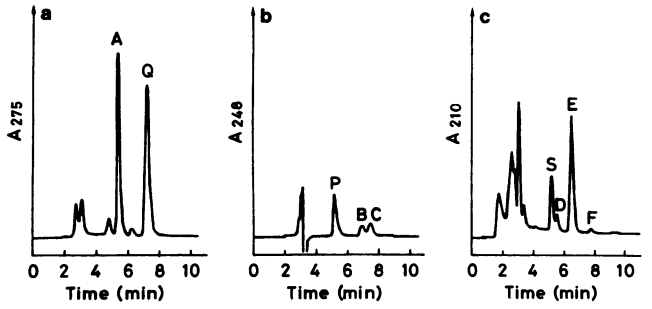

FIG. 2. High-performance liquid chromatography pattern of the isoprenoids from *E. coli* cells. (a) Fraction eluted with 6% diethyl ether from the alumina column. A, Ubiquinone-8; Q, ubiquinon-10 (internal standard). (b) Fraction eluted with 2% diethyl ether from the alumina column. B, Demethylmenaquinone-8; C, menaquinone-8; P, phylloquinone (internal standard). (c) Fraction eluted with chloroform-methanol (1:4, vol/vol) from the silicic acid column. D, Decaprenyl phosphate; E, undecaprenyl phosphate; F, dodecaprenyl phosphate; S, solanesyl phosphate (all-*E*-nonaprenyl phosphate) (internal standard).

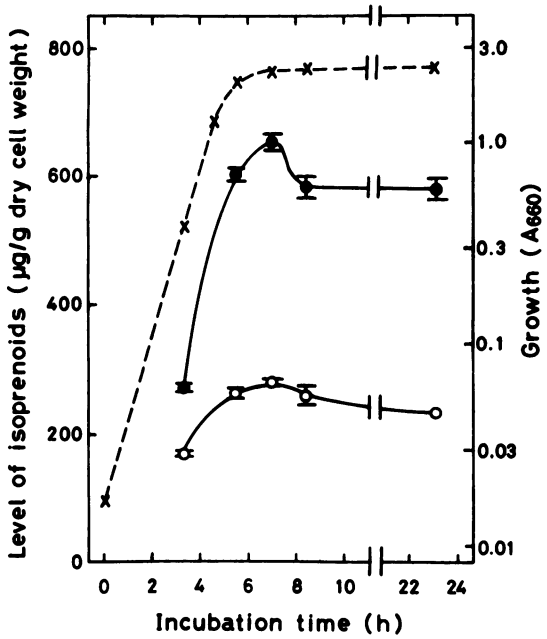

FIG. 3. Levels of ubiquinone-8 and undecaprenyl phosphate in *E. coli* cells during growth. GP1009 was cultivated in L broth at 30°C. Growth (×) was monitored by measuring A_{660}. Levels of ubiquinone-8 (●) and undecaprenyl phosphate (○) were determined as described in Materials and Methods.

TABLE 2. Isoprenoids levels in strains GP1009 and GP1011[a]

Strain	Temp (°C)	Level (μg/g [dry wt] of cells)[b]						
		UQ-8	MK-8	DMK-8	C_{50}-P	C_{55}-P	C_{60}-P	Total Pol-P
GP1009 (*ispA*$^+$)	30	826 (112)	31.7 (2.4)	11.7 (9.0)	21.5 (4.7)	426 (43)	21.7 (4.8)	469 (52)
	42	878 (72)	11.3 (3.3)	1.7 (2.4)	137.4 (7.1)	659 (81)	31.6 (3.1)	828 (83)
GP1011 (*ispA*)	30	846 (90)	38.3 (0.9)	12.7 (11.1)	29.6 (3.4)	540 (29)	29.3 (3.4)	599 (27)
	42	583 (67)	8.0 (0.8)	5.3 (3.9)	67.7 (8.7)	823 (111)	38.3 (10.1)	929 (119)

[a] Values shown represent averages of results obtained from three assays, and those within parentheses are standard deviations.
[b] Abbreviations: UQ-8, ubiquinone-8; MK-8, menaquinone-8; DMK-8, demethylmenaquinone-8; C_{50}-P, decaprenyl phosphate; C_{55}-P, undecaprenyl phosphate; C_{60}-P, dodecaprenyl phosphate; Pol-P, polyprenyl phosphates.

pounds) and were presumed to be sugar carrier lipids (decaprenyl phosphate, undecaprenyl phosphate, and dodecaprenyl phosphate, respectively).

Because the levels of the major isoprenoids were shown to be nearly constant in the period from the late logarithmic phase to the stationary phase (Fig. 3), we compared the level of isoprenoid in the mutant with that in the wild-type strain, using cells in the early stationary phase. Thus, 100 ml of Davis minimal medium inoculated with an overnight culuture was shaken for 8 h for cultivation at 42°C or for 9 h for cultivation at 30°C. The isoprenoid levels in GP1009 and GP1011 grown as described above are shown in Table 2. The ubiquinone level in GP1011 (*ispA*) grown at 42°C was 66% of that in GP1009 (*ispA*$^+$), while the level in GP1011 grown at 30°C was nearly equal to that in GP1009. The levels of menaquinone and demethylmenaquinone were low and almost the same in both strains, although demethylmenaquinone levels were highly erratic. The level of total polyprenyl phosphates was slightly higher in GP1011 than in GP1009 at 30°C and almost the same in both strains at 42°C, despite the fact that in vitro activity of FPP synthase was lower in GP1011 than in GP1009 at both temperatures.

DISCUSSION

JE11046, one of the strains isolated in the screening, had temperature-sensitive FPP synthase, showing no growth at 42°C. The mapping of the mutant gene revealed that the temperature-sensitive growth was not due to temperature sensitivity of enzyme activity. The mapped gene, *ispA*, was presumed to be a structural gene of FPP synthase for the following three reasons: (i) partially purified FPP synthase from the mutant showed a temperature sensitivity higher than that of the wild-type enzyme; (ii) FPP synthase of the wild-type strain was not inhibited by the addition of the enzyme fraction of the mutant; (iii) the *ispA* mutation was recessive to the wild-type *ispA*$^+$ allele. This paper seems to be the first one reporting the mapping of a bacterial gene involved in the condensation of IPP with allyl diphosphates in isoprenoid synthesis on the chromosome.

Although the in vitro activity of FPP synthase of the mutant was markedly lower than that of the wild-type strain, the levels of isoprenoids in the mutant grown at 42°C were nearly the same as those in the wild-type strain, with an exception of the moderately lower level of ubiquinone in the mutant. A similar phenomenon has been reported for *E. coli* mutants having defective enzymes involved in glycerophospholipid synthesis (21). Such a phenomenon may be explainable in terms of the idea either that FPP synthase is not the rate-limiting enzyme in isoprenoid synthesis or that it is stabilized by the intracellular environment.

Although the level of ubiquinone in the mutant grown at 42°C was lower than that in the wild-type strain, the mutant showed a growth rate similar to that of the wild-type strain. This suggests that the level of ubiquinone in the wild-type strain was not so low as to limit growth.

The level of sugar carrier lipid in the mutant grown at 42°C was nearly the same as that in the wild-type strain, whereas the level of ubiquinone was lower in the mutant, as described above. It is attractive to consider that the biosyntheses of ubiquinone and sugar carrier lipid are regulated differently through the two key enzymes, octaprenyl diphosphate and undecaprenyl diphosphate synthases, respectively. Increased IPP, which is caused, for example, by a decrease in the flow of IPP to FPP due to the lowering of FPP synthase activity, is considered to be utilized by the latter enzyme (K_m for IPP, 29 μM) in preference to the former enzyme (K_m for IPP, 2.5 μM) (13). The recent development of measurement of intracellular concentrations of IPP and FPP has pointed out the possibility that the concentration of IPP is an important factor to influence metabolic flow to various isoprenoids (5).

In *Bacillus subtilis* (25, 26) and *Micrococcus luteus* (22, 23), the existence of two respective synthases supplying short-chain prenyl diphosphates as a substrate to the two kinds of long-chain prenyl diphosphate synthases has been reported. No synthase supplying short-chain prenyl diphosphate other than FPP synthase has been reported for *E. coli* (13), but the possibility of its existence cannot be excluded. The cloning of the *ispA* gene and the attempt to isolate the null mutant by disruption of the gene will be effective for elucidation of the role of FPP synthase.

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