

Isolation and Characterization of Isoprene Mutants of *Escherichia coli*

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Isoprenoid compounds are found in all organisms. In *Escherichia coli* the isoprene pathway has three distinct branches: the modification of tRNA; the respiratory quinones ubiquinone and menaquinone; and the dolichols, which are long-chain alcohols involved in cell wall biosynthesis. Very little is known about procaryotic isoprene biosynthesis compared with what is known about eucaryote isoprene biosynthesis. This study approached some of the questions about isoprenoid biosynthesis and regulation in procaryotes by isolating and characterizing mutants in *E. coli*. Mutants were selected by determining their resistance to low levels of aminoglycoside antibiotics, which require an electron transport chain for uptake into bacterial cells. The mutants were characterized with regard to their phenotypes, map positions, enzymatic activities, and total ubiquinone content. In particular, the enzymes studied were isopentenyl diphosphate Δ -isomerase (EC 5.3.3.2), farnesyl diphosphate synthetase (EC 2.5.1.1), and higher prenyl transferases.

Isoprenoid compounds are ubiquitous in nature (21, 22). They are characterized by the presence of the basic five-carbon isoprene unit (Fig. 1) which serves as a fundamental building block for all compounds in this class. Although the end products of isoprene biosynthesis are as diverse as rubber, cholesterol, and ubiquinone, the early part of the pathway has been found to be similar in all organisms studied thus far. Research in our laboratory has primarily focused on the enzymatic mechanisms involved in the conversion of isopentenyl diphosphate (IPP) to squalene (Fig. 1) in fungal, plant, and avian systems. This project was undertaken in order to study the biosynthesis of isoprenes in procaryotic systems. Procaryotes offer the immediate advantage of being amenable to both modern molecular techniques as well as classical genetic techniques, and at the same time they pose interesting questions with regard to the regulation of isoprene biosynthesis.

There are three distinct branches of the pathway in noncarotenogenic bacteria such as *Escherichia coli*: the modification of tRNA (5); dolichols, which are carbohydrate carriers in cell wall biosynthesis (1, 22); and the respiratory quinones ubiquinone and menaquinone (Fig. 2). tRNAs with codons beginning with uracil are modified at A-37 by the addition of a single isoprene unit to the N-6 position of the adenosine. The gene *miaA*, which codes for this prenyl transferase (PT), is expressed at very low levels (5), and as a consequence this reaction is a minor component of the pathway. Dolichols and the respiratory quinones are the major end products in procaryotic isoprene biosynthesis. The biosynthesis of these two classes of compounds diverges after the formation of farnesyl diphosphate (FPP). Dolichol synthesis requires the *cis* addition of eight isoprene units to FPP to give C₅₅ polyprenyl diphosphate. The side chains in respiratory quinones require the *trans* addition of 2 to 11 isoprene units to FPP to yield an overall chain length of 5 to 14 units for menaquinones (MK-*n*, where *n* is the number of isoprene units) and 7 to 14 units for ubiquinones (Q-*n*) (8). The aromatic nucleus is alkylated after the chain is fully formed. In *E. coli*, the chain length of the quinones can vary,

but the major components consist of eight isoprene units for both ubiquinone (Q-8) and menaquinone (MK-8) (9).

From the elegant work of Gibson (14, and references therein) the biosynthesis of ubiquinone from chorismate is well understood. Likewise, the work of Young (32) on menaquinone has defined the pathway of the major respiratory quinone for the anaerobic state. Both classes of quinones were recently the subject of a comprehensive review (4). One notable exception in these studies was the lack of information on isoprene chain formation. The previous studies served as an excellent starting point for this study, which focuses on the events in the pathway beginning with the isomerization of IPP to dimethylallyl diphosphate and leading up to the formation of the *all-trans*-polyprenyl diphosphate and the subsequent addition of this chain to the aromatic nucleus in ubiquinone biosynthesis.

Ubiquinone is a major component of the aerobic respiratory chain. It is estimated that there are approximately 50 molecules of ubiquinone for each of the oxidation complexes in the *E. coli* membrane (29). Thus, by focusing on an end product which is produced in relatively large quantities, we were able to detect perturbations in the early part of the pathway. The rationale for the screening process used was to look for mutants that were altered in levels of ubiquinone production but not affected in the aromatic amino acid pathway, thus narrowing the possibility that we would be duplicating previous work.

There were two major considerations in designing an effective screen for this study. First, if we were going to significantly decrease the availability of the side chain precursor for ubiquinone, we might at the same time decrease both menaquinone and dolichol biosynthesis, all three of which should be essential compounds. With that in mind, we screened for *ts* mutants. Second, we wanted to use an initial selection procedure which would allow us to look at large numbers of mutants, followed by a more stringent screen. Gibson (14) used growth on succinate as a sole carbon source for selection. While this method was excellent, as many as 5 to 10% of the mutations found were in ubiquinone biosynthesis (14); it is also a negative selection and requires up to 72 h for cells to grow on this medium. As a result, we

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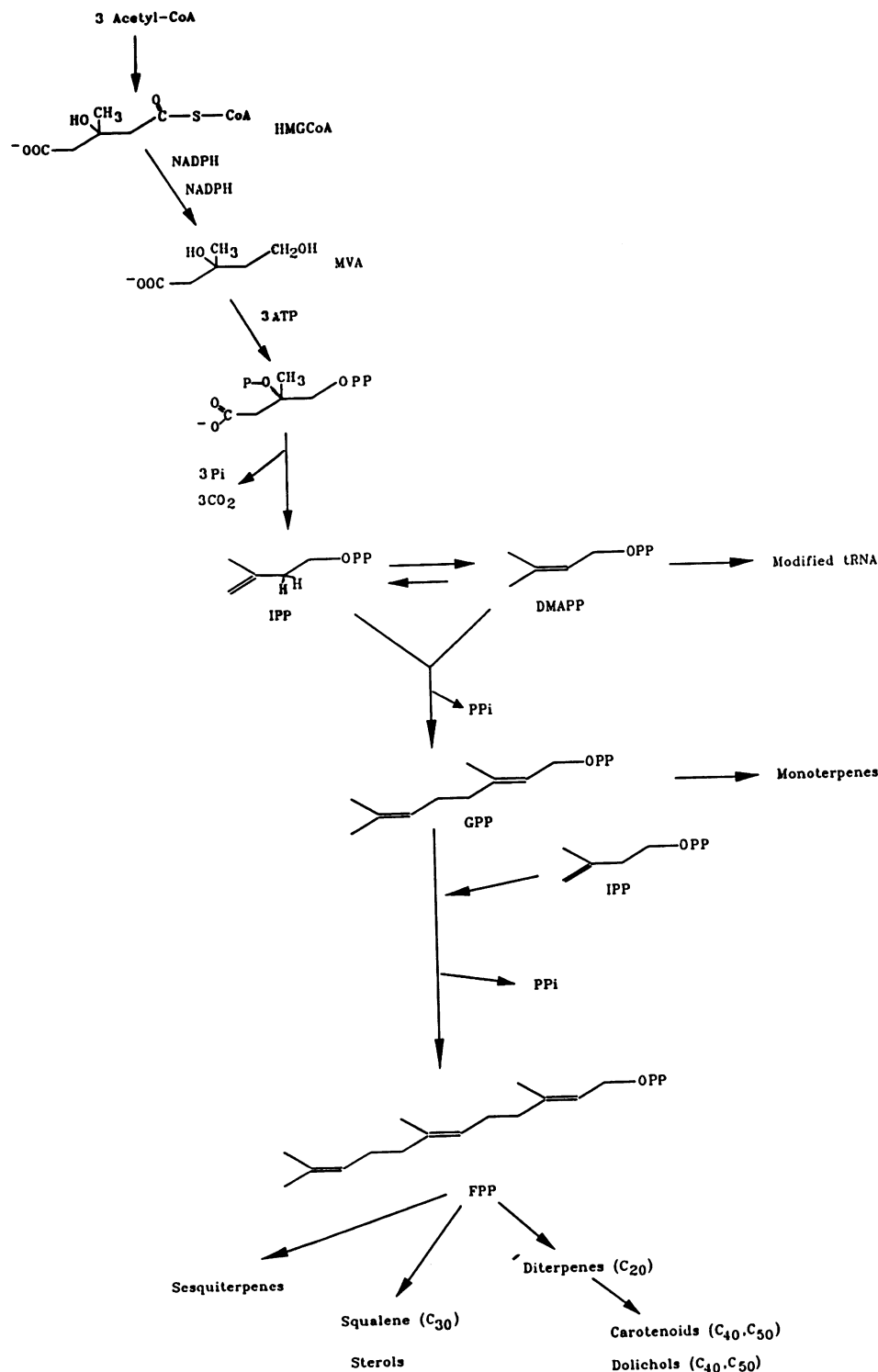


FIG. 1. Isoprene biosynthetic pathway. In most organisms, except possibly *E. coli* (19), isoprene biosynthesis begins with the condensation of three molecules of acetyl coenzyme A (acetyl-CoA) to yield 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA). In all organisms, the pathway from 3-hydroxy-3-methylglutaryl coenzyme A to FPP is similar; at this point the pathway diverges. Abbreviations: MVA, mevalonic acid; OPP, diphosphate group; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate.

chose to use resistance to low levels of aminoglycoside antibiotics as the first step in our selection procedure. This method was previously reported by Taber and co-workers (26) for the isolation of menaquinone mutants in *Bacillus*

subtilis. It takes advantage of the fact that the uptake of aminoglycoside antibiotics requires an energy transport system (10, 27, 28), and one of the major components of this system is ubiquinone.

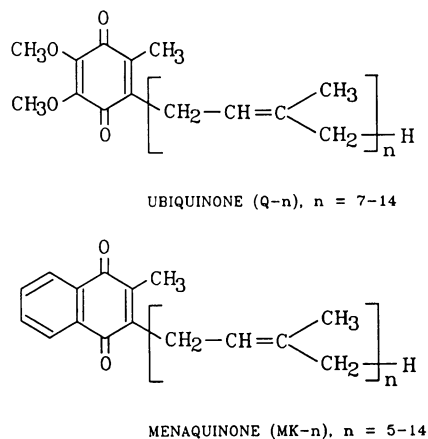


FIG. 2. Structures of the respiratory quinones. Ubiquinone, also known as coenzyme Q, is abbreviated as Q-*n*, where *n* represents the number of isoprene units. The same abbreviation applies to menaquinone, or MK-*n*.

MATERIALS AND METHODS

Chemicals. IPP, geranyl diphosphate, and FPP were prepared by the method of Davisson and co-workers (12). Bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic acid anhydride was synthesized from cyclopentadiene and malic anhydride (13). [1-¹⁴C]IPP was purchased from either Dupont, NEN Research Products (Boston, Mass.) or Amersham Corp. (Arlington Heights, Ill.). The antibiotics (neomycin, kanamycin, gentamicin, and ampicillin) were obtained from Sigma Chemical Co. (St. Louis, Mo.). The coenzyme Q standards (Q-6, Q-7, Q-9, and Q-10) were also purchased from Sigma. Ultrapure Tris was obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) was from Sigma. The mutagens diethyl sulfate (DES), methanesulfonic acid ethyl ester (EMS), and hydroxylamine were from Sigma. All solvents were UV grade (Omnisolve, EM-Science); hexanes were dried over MgSO₄ prior to use. The scintillation cocktail used was either Insta-fluor or Opti-fluor (Packard Instrument Co., Inc., Rockville, Md.).

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. PA3092 and *ts* mutants from the collection of Suzuki et al. (25) were obtained from Rolf Sternglanz. The *E. coli* cosmid library was obtained from Michael O'Connor. The cosmids were prepared by partial *Sau*III digestion of total chromosomal DNA and insertion of ca. 40-kilobase-pair fragments into the *Bam*HI site of pREG153, a low-copy-number (two to three) vector.

All media ingredients were from Difco Laboratories (Detroit, Mich.). The enriched medium used was either LB (17) or PB medium. PB medium consisted of 12 g of tryptone (Difco), 24 g of yeast extract, 5 ml of glycerol, and 0.1 M KPO₄ (pH 7.6) buffer per liter. The minimal medium used was either M9-glucose (17) or M9-succinate medium, which substituted 30 mM sodium succinate for glucose as the sole carbon source. Where indicated, supplements were added to the minimal media (Tables 1 and 2) at the following concentrations: 0.6 mM arginine, 0.1 mM glycine, 0.1 mM histidine, 0.05 mM thiamine, 0.1 mM uracil, 0.1 mM tryptophan, 0.1 mM tyrosine, 0.3 mM methionine, 0.3 mM leucine, 2.0 mM proline, 0.3 mM threonine, 0.3 mM thymine, 4 mM serine, 0.3 mM phenylalanine, 1 μM 4-hydroxybenzoic acid, 0.2%

galactose, 0.2% lactose, and 0.2% mannose. When required, antibiotics were added to the media as follows. Ampicillin was added at a final concentration of 100 μg/ml; neomycin, gentamicin, and kanamycin were prepared as fresh solutions daily and spread onto the plates just prior to use to give final concentrations of 40 μg of neomycin per 25 ml, 20 μg of gentamicin per 25 ml, and 30 μg of kanamycin per 25 ml. The λYM medium used consisted of 10 g of tryptone (Difco), 2.5 g of NaCl, 1 g of yeast extract, and 4 ml of 50% maltose per liter. The SM medium used for the preparation of the lambda lysates consisted of 5.8 g of NaCl, 2 g of MgSO₄, 50 ml of 1 M Tris (pH 7.5), and 5 ml of 2% gelatin per liter.

Mutant isolation and screening. The concentrations of the three antibiotics used in this study (neomycin, kanamycin, and gentamicin) were determined by comparing the relative levels of resistance of W3110, AN162, AN163, AN211, and AN385. A wide range of concentrations was tested initially by using the gradient plate technique of Bryson and Szybalski (6). The final levels of antibiotics were determined by using antibiotic sensitivity disks (diameter, 0.5 in [1.27 cm]; 740-E; Schleicher & Schuell, Inc., Keene, N.H.). Antibiotics were screened at levels of 1, 5, 10, 20, and 30 μg per disk.

Strain W3110 was mutagenized by exposure of exponentially growing cultures to DES, EMS, or NH₄OH by the methods of Miller (18) and Carlton and Brown (7). Mutants were selected by comparing their temperature-sensitive behavior on LB plates at 30 versus 42°C and by determining their resistance to all three aminoglycoside antibiotics at 37°C. Strains with the mutant phenotypes were tested for stability by carrying the strains through four successive transfers and verifying the phenotypes at each stage. Mutants which showed consistent phenotypic behavior were then tested for their inability to grow on M9-succinate plates at 30°C over a period of 72 to 96 h. Mutants which had a phenotype of Nm^r Km^r Gm^r M9-succinate⁻ were then assayed for their enzymatic activities in isomerase, PT, and higher PT (HPT) at 30, 37, or 42°C, as indicated. Strain PA3092 and 120 of the mutants from the *ts* mutant collection of Suzuki et al. (25) were screened for both their temperature sensitivity and their antibiotic resistances, as described above. Mutants in this collection which showed consistent phenotypes were assayed for isomerase, PT, and HPT activities at 30 and 42°C.

Genetic procedures. Collections of F[']-containing strains and Hfr::Tn10 strains were obtained from B. Bachmann and were used to map the mutations as described by Miller (18). Complemented strains resulting from episome transfer were selected for their ability to grow on M9-succinate plates. The cotransduction studies were performed by selecting for growth on M9-glucose-tetracycline and by scoring for growth on M9-succinate and LB-tetracycline.

The cosmid bank was introduced into the mutants strains via lambda transduction (18) by using λNK561 (31). The transduced cells were plated onto LB-ampicillin. Colonies were picked randomly and tested for growth on LB-neomycin, LB-kanamycin, and LB-gentamicin. Colonies which had a phenotype of Ap^r Nm^s Km^s Gm^s were then tested for their ability to grow on M9-succinate medium.

Enzyme assays. Strains were grown in 25 ml of PB medium at 30°C for 5 h after inoculation of 2 to 4% of an overnight culture. Under these conditions the cultures were harvested at the late exponential phase of growth. Cells were collected by centrifugation, washed once with 0.9% saline, weighed to determine a wet cell weight, and suspended in 2.0 ml of assay buffer. The assay buffer consisted of 10 mM PIPES, 1 mM dithiothreitol, 2 mM MgCl₂, 4 mM KF, and 0.01%

bovine serum albumin. Cells were broken by sonication with a Branson model 350 sonifier, output of 2.5, pulsed mode at 70% duty cycle, for three times 30 s. The homogenate was centrifuged at $20,000 \times g$ for 10 min. The cleared supernatant was transferred to a clean tube, and samples were assayed for enzymatic activity. Protein content was determined by using a modified Bradford assay (24).

IPP isomerase assay mixtures contained 6 nmol of [1^{14}C]IPP (10 $\mu\text{Ci}/\mu\text{mol}$), 40 μl of assay buffer, and 30 μl of supernatant in a total volume of 100 μl . The assay mixture was incubated for 10 min at 30, 37, or 42°C and then quenched with 200 μl of methanol-HCl (4:1) and further incubated at the same temperature for 10 min. The assay mixture was extracted with 1.0 ml of ligroine by vortex mixing at low speed for 15 s. A 0.5-ml portion of the ligroine layer was removed and mixed with scintillation cocktail for counting. FPP synthetase (PT) assay mixtures contained 20 nmol of geranyl diphosphate, 4 nmol of [1^{14}C]IPP (10 $\mu\text{Ci}/\mu\text{mol}$), 46 μl of assay buffer, and 30 μl of supernatant in a total volume of 100 μl . The assay for PT was carried out exactly the same as the isomerase assay. The assay mixture for HPT consisted of 20 nmol of FPP, 4 nmol of [1^{14}C]IPP (10 $\mu\text{Ci}/\mu\text{mol}$), 46 μl of assay buffer, and 30 μl of supernatant. The solution was incubated for 30 min and then quenched and extracted as described above. Each strain was assayed several times, and each flask was assayed in duplicate.

The calculations for the enzyme activities were done by the following procedure. The corrected disintegrations per minute were determined by subtracting the background counts of a control assay (no enzyme present) from the disintegration-per-minute reading for each assay. This value was then multiplied by 2 to account for the total disintegrations per minute for that assay. The total protein content of each extract was determined by a modification of the Bradford assay (24) by using bovine serum albumin as a standard. The disintegrations per minute per assay were then divided by the amount of protein (in milligrams) per assay to give the final value. Each strain was assayed several times, and the results of each assay were combined and analyzed with regard to median, standard deviation, and percent error (standard deviation/median). The median value for each assay was used to calculate the ratios.

HPLC assays for ubiquinone. The assay for ubiquinone was based on the methods cited by Collins (8). Strains were grown in 50 ml of PB at 30°C for 5 h after inoculation with 2 to 4% of an overnight culture. Cells were collected by centrifugation, washed once with 0.9% saline, weighed to determine a wet cell weight, and then lyophilized overnight. The dried pellet was extracted in 25 ml of CHCl_3 -methanol (2:1) containing 5 μg of Q-10 as an internal standard by stirring the suspension at room temperature for 2 h; the suspension was protected from light and moisture. The extract was filtered, and solvents were removed in vacuo. The residue was suspended in 1.5 ml of dry hexanes and loaded onto a prewashed cartridge (Florisil Sep-Pak; Waters Associates, Inc., Milford, Mass.). The cartridge was then washed three times with 1 ml of dry hexanes, which was followed by elution of lipophilic material with three portions of 1 ml of dry hexanes-ethyl acetate (3:1). Solvents were evaporated under nitrogen, and the tube was sealed with Parafilm and stored in the dark until high-pressure liquid chromatographic (HPLC) analysis. HPLC was done on a Waters instrument (model 6000A pump; Lambda-Max model 480 variable-wavelength detector, and data module). An octadecylsilane, 5 μm Hypersil column (24 by 0.38 cm) was used in tandem

TABLE 1. Bacterial strains used in this study

Strain	Description	Source or reference
W3110	F ⁻ λ ⁻	Bachmann (3)
AN162	Hfr <i>metB oct-401</i>	I. G. Young
AN163	Hfr <i>metB oct-402</i>	I. G. Young
AN211	F ⁻ <i>metB menA406</i>	Young (32)
AN385	F ⁻ <i>ubiA420 Str^r</i>	Wallace (30)
PA3092	F ⁻ <i>thr leu thi argH thyA his trp lacY1 mtl xyl malA mel tonA str supE</i>	Sternglanz (25)

with a guard column (Waters) containing a C-18 $\mu\text{Bondapak}$ cartridge. The mobile phase of methanol-1-chlorobutane (100:15) was used at a flow rate of 1.5 ml/min. The eluant was monitored at 270 nm. When necessary, fractions were collected at 1-min intervals. Similar fractions from repeated injections were combined and the solvent was removed in vacuo. The sample was then reinjected onto either the same column or a 10- μm C-18 $\mu\text{Bondapak}$ column (Waters), and the fractions were collected again as described above.

Miscellaneous procedures. Mass spectra were obtained on a double-focusing, high-resolution mass spectrometer (VG Micromass 7070) with a data system (VG 2000). Mass spectra of Q-8 (EI, 70 eV, 250°C; m/z [intensity]): 726 (4.9, M⁺), 235 (76.6), 197 (38.1), 69 (100.0). Mass spectra of UN-1 (EI, 17 eV, 250°C; m/z [intensity]): 716 (61.9, M⁺), 633 (39.3), 410 (100.0), 225 (86.4), 187 (64.8), 135 (69.9), 81 (74.6), 69 (69.9). The peaks at 716, 225, and 187 can be assigned to MK-8. High-resolution mass spectra of the molecular ion, 716, yielded a measured mass that agreed to within 1.6 millimass units (measured mass, 716.55483; proposed mass, 716.55322 for $\text{C}_{51}\text{H}_{72}\text{O}_2$). The base peak at 410 could not be assigned to MK-8, and as determined by the mass spectral experiment, it was a distinct compound from MK-8.

RESULTS

Isolation of mutants. The conditions for the antibiotic resistance screen were established by using strain W3110 and the mutants obtained from I. G. Young, including AN162, AN163, AN211, and AN385 (Table 1). Optimal antibiotic concentrations were determined by the gradient plate technique in order to test a wide range of concentrations, followed by the antibiotic sensitivity disk assay described above. The mutants, but not the parental strain W3110, were resistant at the lower levels tested, with resistance decreasing in the order neomycin, kanamycin, and gentamicin. Based on these results, we decided to use the following concentrations: neomycin, 40 μg per plate; kanamycin, 30 μg per plate; and gentamicin, 20 μg per plate. The final levels were confirmed with a trial experiment by using the mutants of I. G. Young under mutagenesis conditions. The three chemical mutagens DES, EMS, and NH_4OH were chosen to induce a variety of mutations. Following mutagenesis, selection for mutants required that the strain show a Ts phenotype and be resistant to all three antibiotics. Any strain which showed consistent phenotypic behavior through four successive transfers was assigned a strain number and stored as a stable mutant. These strains were tested later for their inability to grow on M9-succinate medium. All mutant strains which were tested further for enzyme activity and total ubiquinone content had the Nm^r Km^r Gm^r M9-succinate⁻ phenotype. Strains with the Ts

TABLE 2. Mutant characteristics

Strain	Phenotype ^a					Auxotrophic markers	Approx. map position
	Ts	Nm'	Km'	Gm'	M9-succinate		
MS5015	-	++	++	++	++	None	NA ^b ; 80% cotransduced to <i>purE</i>
MS5016	-	++	++	++	++	None	13.5-30 min; 50% cotransduced to <i>purE</i>
MS5031	-	+	+	+	+	None	13.5-17 min; 4% cotransduced to <i>purE</i>
MS5041	+	*	+	*	++	Glycine	34-44 min
MS5048	-	*	+	*	++	None	34-44 min; 95% cotransduced to <i>srlC</i>
AN162	-	++	++	++	++	Methionine	34-44 min; 20% cotransduced to <i>srlC</i>
AN163	-	++	++	++	++	Methionine	17-30 min

^a Phenotypes were determined by growth on the media indicated in the text. Abbreviations: -, the indicated phenotype is absent; *, the indicated phenotype is slight; +, the indicated phenotype is strong; ++, the indicated phenotype is very strong.

^b NA, F' data not available.

phenotype were not consistently present, and this phenotype was later shown by enzyme assay and complementation not to be associated with the enzymes we were testing.

Summary of stable mutants. We ran 17 different mutagenesis experiments with strain W3110, of which 12 used DES, 3 used EMS, and 2 used NH₄OH. DES was by far the most effective mutagen for these experiments, whereas NH₄OH did not yield any mutants. In the 17 experiments, a total of 48 mutants (ca. 0.1% of the total number of mutants isolated) were both *ts* and antibiotic resistant. Of these mutants, 20 (42%) showed a stable phenotype. After preliminary enzyme assays, nine mutants were selected for further testing. The phenotypes of three of these mutants later reverted.

We also screened 120 mutants from the *ts* mutant library of Suzuki et al. (25). In this collection we found 27 (22%) mutants that were *ts* and antibiotic resistant. Of these, eight were selected for further analysis because of their strong antibiotic-resistant phenotype, as determined by their growth characteristics on the different antibiotic-containing media.

Genetic analysis. The MS strains, along with strains AN162 and AN163, were analyzed for auxotrophic requirements (11). While we did not expect auxotrophy as a consequence of the isoprene mutations, it could result from a second site mutation, or it could occur as a result of a mutation in the aromatic portion of the pathway. The mutations in these strains were also mapped to determine their approximate positions by using an F' collection from B. Bachmann as described by Low (16). The regions defined by F' complementation were narrowed by using selective cotransduction studies. In addition we used the λ library of *E. coli* of Kohara et al. (15) for fine mapping of the mutations. The mutants were analyzed for their phenotypes at the end of this study; and these data, along with the auxotrophic requirements and map positions, are presented in Table 2. It should be noted that initially all of the mutants showed a strong antibiotic resistance phenotype. The *ts* mutant library of Suzuki et al. (25) was analyzed only with respect to the Ts and antibiotic resistance phenotypes.

Enzyme assays. We performed assays for three different enzymatic activities in the mutants: IPP isomerase, FPP synthetase (PT), and HPT (Fig. 3).

Initial experiments were conducted to determine the optimal conditions for enzymatic activity; and it was found that the activities of all three enzymes responded differently to buffers, temperature, and length of incubation. The conditions given above were a compromise so that all three enzyme activities could be obtained from the same crude cell extract. Both PT and HPT activities showed a much greater response under different conditions than did isomerase activity.

Isomerase activity was distinctly lower than PT or HPT activity, and several precautions had to be taken in interpreting the results of the isomerase assay. In particular, it was necessary to eliminate phosphatase activity, which would give higher counts in the assay due to the hydrolysis of IPP to give a ligroine-soluble alcohol without isomerization of the double bond. Phosphatase activity was determined by quenching a tube with 0.2 ml of ice water, which was then incubated at 0°C for 10 min before the ligroine extraction. Under the conditions of the assay (4 mM KF in the buffer), we did not detect the hydrolysis of IPP.

The MS strains were initially assayed at both 30 and 42°C after growth at 30°C, to screen for *ts* mutant enzyme activity. In all instances activities were higher at 42°C than at 30°C, and parallel behavior was found for strain W3110. The data presented in Table 3 were determined at 37°C. The *ts* mutant collection was assayed at both temperatures, and the results are summarized in Table 4.

For each enzyme assayed, the disintegrations per minute per milligram of protein for the mutant was compared with that of the wild type to give the ratios cited in Tables 3 and 4. The MS strains and strains AN162 and AN163 gave consistent results: 64% of the assays had less than 11% deviation from the mean, 33% of the assays had between 11 and 15% deviation, and 3% had greater than 15% deviation. The largest variations were seen for flasks containing the same strain. In the *ts* mutant collection, 65% of the assays had less than 11% deviation, 20% had between 11 and 15% deviation, and 15% of the assays had greater than 15% deviation.

The assay for isomerase activity gave incorporation of the label that was only twofold above background for the wild-type strain. Thus, only those strains that showed greater than 50% reduction in isomerase activity were considered to be isomerase mutants. The PT assay may have contained some HPT activity, although PT activity was typically much greater than that of HPT. In addition, the HPT assay did not distinguish between *trans*- and *cis*-condensing enzymes, which produce undecaprenyl diphosphate and bacterial dolichyl diphosphate, respectively. The two different enzymatic activities detected by the HPT assay could not be determined without doing product studies (Fig. 3). In order to distinguish the different enzymatic activities of isomerase, PT, and HPT, the conversions were maintained at less than 10%.

By using these criteria, a review of the results (Tables 3 and 4) indicates that we identified four isomerase mutants, MS5031, MS5048, AN162, and AN163 (*ts* mutants 25 and 62 were considered borderline mutants). Likewise, several good PT mutants (MS5031, MS5048, and AN162, and *ts* mutants 25, 38, 51, 62, and 301) were found. In the case of

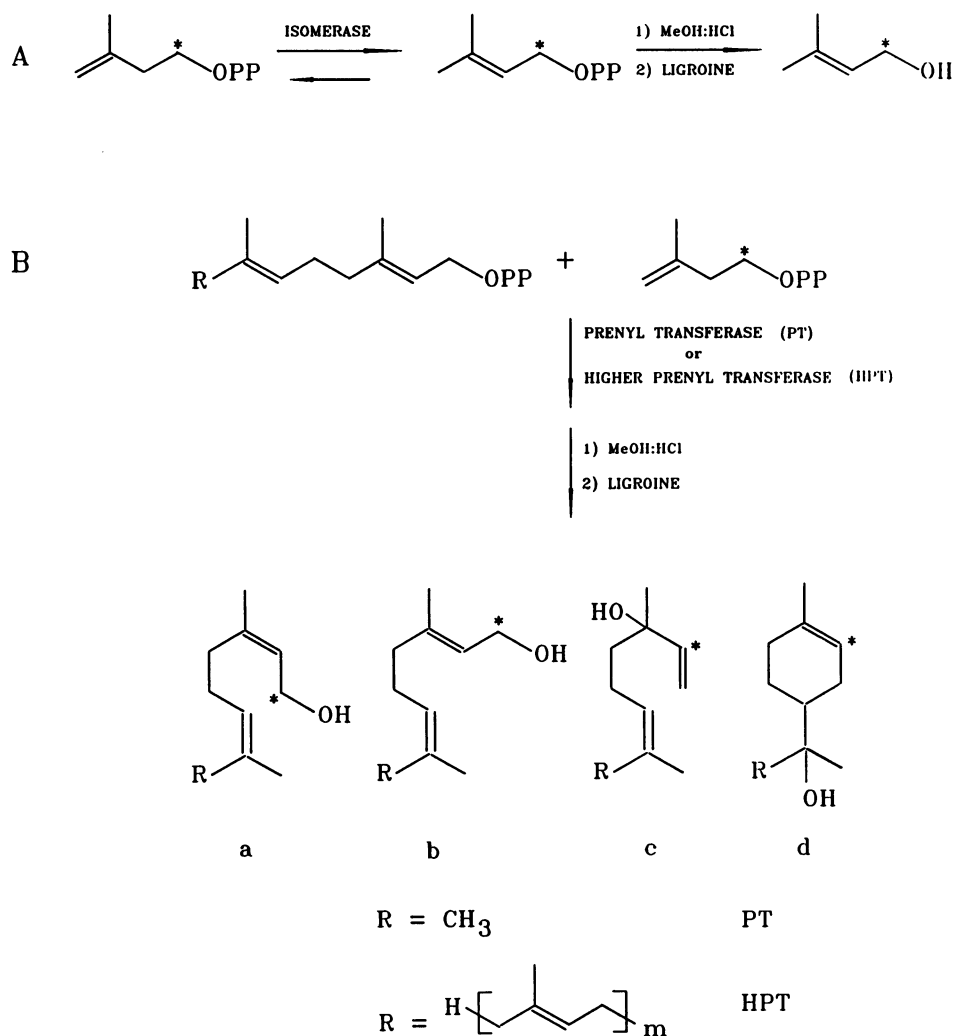


FIG. 3. Enzyme assays for IPP isomerase (A) and PT and HPT (B) were performed according to the acid lability assay. For the HPT assay, the substrate was FPP ($m = 1$), and the product was $m \geq 4$ in either the *trans* or the *cis* (data not shown) conformation. The acid hydrolysis quench cleaved an allylic diphosphate bond, but not a homoallylic diphosphate bond, thus allowing the distinction between dimethylallyl diphosphate or higher prenyls, and IPP. The hydrolysis of geranyl diphosphate (and higher diphosphates) has been shown previously (23) to yield the four products indicated, in which the total product was 3% (a), 27% (b), 53% (c), and 17% (d). Abbreviations: OPP, diphosphate; MeOH, methanol. *, Location of radiolabel.

HPT, we had far fewer mutants and no strong ones. AN162 and AN163 both showed higher HPT activity than the wild type, while *ts* mutants 33 and 62 had lower activities.

Complementation with the cosmid library. The cosmid library, which was packaged in λ NK561, was introduced into the nine MS mutants and AN162 and AN163. After repeating the infection four times, we were able to isolate cosmids which complemented strains for seven of the nine MS strains and strains AN162 and AN163. MS5015 and MS5016 were refractory to complementation by this cosmid bank. AN162 gave only one isolate that was phenotypically complemented. All of the new strains generated by the introduction of the cosmid bank had a phenotype of temperature insensitivity $\text{Nm}^s \text{Km}^s \text{Gm}^s$ and were able to grow on M9-succinate medium. MS5041 was not strong either phenotypically or by the enzyme assay, and the respective complemented strain was not followed. All of the other strains were assayed for enzymatic activity (Table 3) and total ubiquinone content (Table 5).

Complementation of the MS strains was interesting in that each one showed a different pattern of complementation (see Tables 3 and 7). MS5048(p5330) showed an overall twofold increase for each of the three enzymes. AN162(p5331) had a twofold increase in isomerase and PT activities but showed a twofold decrease in HPT activity, which was originally higher than wild type. MS5031(p5336) had a dramatic nine-fold increase in isomerase activity, with very little change in either PT or HPT activity, thus suggesting that either MS5031 has a mutation in a regulatory region or the plasmid carries a regulatory region, since the copy number of the cosmid was two to three. Isomerase activity increased 1.5-fold in AN163(p5341), while no change was seen for PT activity and HPT activity decreased 1.5-fold.

HPLC assays of total ubiquinone content. All of the strains in this study were assayed for total ubiquinone content by a modification of a previously described procedure (8). Each strain was assayed at least four times. Precautions were taken during the workup of cultures to avoid degradation of

TABLE 3. Enzyme levels in MS mutants relative to that in the wild type

Strain	Enzyme activity ^a		
	Isomerase	PT	HPT
MS5015	0.95	0.94	1.04
MS5016	0.86	0.71	1.24
MS5031	0.33	0.38	1.10
MS5031(p5336)	2.87	0.48	1.36
MS5041	0.75	0.95	1.06
MS5048	0.50	0.62	0.89
MS5048(p5330)	0.95	1.11	1.34
AN162	0.38	0.42	1.37
AN162(p5331)	1.02	0.74	0.64
AN163	0.51	1.01	1.61
AN163(p5341)	0.74	1.01	0.94

^a Enzyme activities were determined by standard assays (see the text) and are expressed as a ratio of the levels in the indicated mutant strain relative to the levels in the wild-type strain W3110. All strains were grown at 30°C and assayed at 37°C.

ubiquinone from moisture, air oxidation, and UV light. In addition, Q-10 was added as an internal standard to each sample. Loss of the internal standard was determined by comparison with a standard curve, and this factor was used to correct the peak areas of the naturally occurring quinones, assuming that all quinones underwent similar losses during workup. The total ubiquinone content was determined by dividing the corrected peak area by the wet cell weight (milligrams of cells) of each sample. Despite these precautions, the samples showed a considerable variation, with the standard deviations being up to 35% of the mean values.

Peaks were identified by comparing their elution profiles with the standards for Q-6, Q-7, Q-9, and Q-10. A standard sample of Q-8 was not readily available, and the Q-8 peak was identified by its elution profile relative to those of the other standards (8) and by mass spectral analysis of the isolated compound. The HPLC profiles of the MS mutants and AN162 and AN163 showed low levels of Q-6 and Q-7 relative to the level of Q-8 and no Q-9. Some of the *ts* mutant collection and all of the strains which contained cosmids showed various levels of Q-9, in addition to the major quinone Q-8. In all of the samples there was a peak which eluted between Q-9 and Q-10. This peak was named UN-1. High-resolution mass spectral analysis indicated that this

TABLE 4. Enzyme levels in *ts* mutant strains relative to those in the parental strain

Strain ^a	Enzyme activity ^b					
	Isomerase		PT		HPT	
	30°C	42°C	30°C	42°C	30°C	42°C
25	0.61	0.52	0.62	0.56	0.87	0.61
33	0.84	0.81	0.76	0.83	0.73	0.60
38	0.93	0.66	0.65	0.69	1.02	0.76
51	1.09	1.07	0.50	0.54	0.80	0.79
62	0.55	0.60	0.69	0.64	0.71	0.74
101	0.81	0.79	0.44	0.47	0.72	1.03
112	1.58	1.14	0.92	0.76	1.15	0.90
301	1.13	1.13	0.37	0.35	0.90	0.96

^a The *ts* mutant strains of Suzuki et al. (25).

^b Enzyme activities were determined by standard assays (see the text) and are expressed as a ratio of the levels in the indicated mutant relative to the levels in the parental strain PA3092. Strains were grown at 30°C and assayed at both 30 and 42°C.

TABLE 5. Levels of quinones in MS mutants

Strain	Level of the following quinones:		
	Q-8 (mutant/ wild type) ^a	UN-1 (mutant/ wild type) ^a	Q-8/ UN-1 ^b
W3110	1.0	1.0	4.1
MS5015	0.1	6.2	0.1
MS5016	0.1	0.1	0.9
MS5031	0.4	0.1	19.1
MS5031(p5336)	1.6	1.3	5.0
MS5041	1.3	1.1	4.9
MS5048	0.8	0.2	14.7
MS5048(p5330)	0.9	0.8	4.3
AN162	0.1	0.2	2.9
AN162(p5331)	0.6	0.4	6.0
AN163	1.3	0.4	15.6
AN163(p5341)	3.5	1.3	10.9

^a Levels of quinone in the indicated mutant strain relative to that in the wild type, W3110. Calculations are explained in the text.

^b Ratio of Q-8 to UN-1 within the indicated strain.

peak contained at least two compounds, one of which was identified as MK-8. We were not able to separate the components by HPLC methods. A sample of representative HPLC profiles is shown in Fig. 4, and a summary of the relative levels of Q-8 and UN-1 is presented in Tables 5 and 6.

The results of the HPLC assays for Q-8 content (Tables 5 and 6) confirmed the results from the enzyme assays and the antibiotic resistance screen. Strains MS5015 and MS5016 had a very strong phenotype (Table 2), but neither showed a significant decrease in the enzyme activities for isomerase, PT, or HPT. However, both had a 10-fold decrease in the levels of Q-8. The implication is that we altered another enzyme in the pathway defined by Gibson (14). MS5031, which gave a 60% decrease in both isomerase and PT activities, had approximately 60% less Q-8. MS5048, which also had reduced levels of isomerase and PT activity, had slightly decreased levels of Q-8 and significantly decreased levels of UN-1. AN162, with decreased levels of isomerase and PT activity and an increased level of HPT activity, had a 10-fold decrease in Q-8. AN163 was unique in that isomerase levels were decreased, while HPT levels were elevated. The HPLC assay for AN163 showed slightly elevated levels of Q-8 and decreased levels of UN-1. Within the *ts* mutant collection, the strongest mutants were *ts* 33, *ts* 38, *ts* 51, and *ts* 101. Mutant *ts* 101 was interesting in that the levels of Q-8 decreased, and there was no detectable UN-1.

DISCUSSION

The isolation of isoprenoid mutants took advantage of two observations. The major end products of this pathway are ubiquinone and menaquinone, both of which contain isoprenoid side chains; and aminoglycoside antibiotics require active electron transport for uptake (10, 27, 28), of which the major component of the *E. coli* electron transport chain is ubiquinone (29). This selection was previously used by Taber and co-workers (26) to isolate menaquinone mutants in *B. subtilis*. In early work by Gibson (14), ubiquinone mutants were screened for their inability to grow on succinate as a sole carbon source. The work of Gibson (14) focused on the assembly of the aromatic nucleus and did not attempt to address the synthesis of isoprenoid side chains. Although the succinate screen was not used initially in this

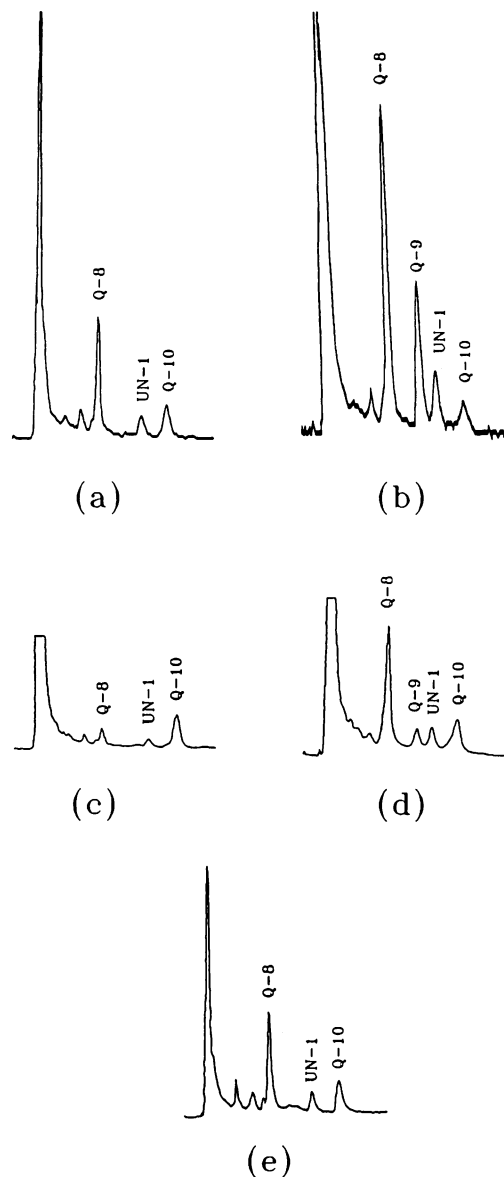


FIG. 4. Traces of isolated quinones determined by HPLC. The relevant peaks were labeled in each trace. The traces were normalized by using the Q-10 peak. Samples were as follows: MS5031 (0.5 absorbance units, full scale [AUFS]) (a), MS5031(p5336) (0.5 AUFS) (b), AN162 (0.1 AUFS) (c), AN162(p5331) (0.1 AUFS) (d), and W3110 (0.5 AUFS) (e).

study, the procedure was used to verify mutants isolated from the antibiotic resistance selection procedure.

Reduction in the availability of the isoprenoid side chain might alter both ubiquinone and menaquinone production, as well as dolichol biosynthesis, if the mutation occurred prior to FPP synthesis, which is a branch point in the pathway. Wallace and Young (30) have constructed a *ubiA menA* double mutant, but have noted that the growth of the strain is severely affected by the double mutation. It was not clear whether it is possible to isolate a double mutant from a mutagenized culture by simple selection techniques. Thus, we looked for conditional lethal mutants by selecting for a Ts phenotype in our mutagenesis experiments and in the *ts* mutant library of Suzuki et al. (25). Considering the variety

TABLE 6. Levels of quinones in *ts* mutant strains

Strain ^a	Level of the following quinones:		
	Q-8 (mutant/parent) ^b	UN-1 (mutant/parent) ^b	Q-8/ UN-1 ^c
PA3092	1.0	1.0	2.7
<i>ts</i> 25	1.1	1.3	2.3
<i>ts</i> 33	0.5	1.0	1.3
<i>ts</i> 38	0.9	0.5	4.9
<i>ts</i> 51	0.2	0.1	8.0
<i>ts</i> 62	0.7	0.7	2.7
<i>ts</i> 101	0.4	ND ^d	
<i>ts</i> 112	0.8	0.4	5.0
<i>ts</i> 301	1.2	0.9	3.8

^a The *ts* mutant strains of Suzuki et al. (25).

^b Levels of quinone in the indicated mutant strain relative to that in the parental strain PA3092. Calculations are explained in the text.

^c Ratio of Q-8 to UN-1 within the indicated strain.

^d ND, Not detected.

of mutagens used and the degree to which the cultures were mutagenized (W3110 was mildly mutagenized with either EMS or DES; PA3092 was heavily mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), we expected to find conditional lethal mutants exhibiting severe decreases in enzymatic activity. However, we were unable to find a mutant that was temperature sensitive in isomerase, PT, or HPT. In the best cases, we identified mutants that had lost ca. 60% of the enzyme activity (Tables 3 and 4).

Since we were searching for tightly linked activities in the metabolic pathway, it was possible that they were located within a single operon. The strains in this study appeared to have mutations which mapped to two distinct regions of the chromosome. AN162 and MS5048 mapped between 34 and 44 min by episome transfer and were shown to be linked to *sr/C* at 58 min. The other strains had mutations which mapped between 13.5 and 30 min, as determined by F' complementation; and in the cases of MS5015, MS5016, and MS5031, they were shown to be linked to *pure* at 12 min. The strain which showed the greatest variation in the mapping studies was MS5031, probably because of a second mutation which had the same phenotype. From the results of the enzyme and HPLC assays, we know that the levels of the enzymes of interest as well as the total ubiquinone content for these strains were altered; but until we obtain smaller subclones and fine map the region, we cannot say for certain which gene, regulatory region, or both that we mutated.

We were able to restore both enzymatic activity (Table 3) and total ubiquinone content (Table 5) to most of our mutants by introduction of an *E. coli* cosmid bank. Unfortunately, the two mutants with distinct phenotypes, MS5015 and MS5016, were refractory to complementation by the cosmid bank. Complementation of the *ts* mutant collection of Suzuki et al. (25) was not straightforward, because the parental strain PA3092 had several auxotrophic requirements and secondary mutations were created by heavy mutagenesis (24). Work is now under way to subclone the cosmids which were subsequently able to complement the mutations. Initial results show that the cosmids from MS5031, MS5048, AN162, and AN163 have unique restriction patterns (M. M. Sherman, unpublished data), suggesting that the inserts are from distinct regions of the chromosome, which confirm the mapping results.

We were able to identify potential mutations in the structural or regulatory genes for isomerase, PT, and HPT by

TABLE 7. Changes in the enzyme activities and levels of quinones in MS mutants^a

Strain	Change in enzyme activity:			Change in quinone level		
	Isomerase	PT	HPT	Q-8	UN-1	Q-8/UN-1
MS5015	—	—	—	↓	↑	↓
MS5016	—	↓	↑	↓	↓	↓
MS5031	—	↓	—	↓	↓	↑
MS5031(p5336)	↑	↓	↑	↑	↑	—
MS5041	↓	—	—	↑	—	—
MS5048	↓	↓	—	—	↓	↑
MS5048(p5330)	—	—	↑	—	—	—
AN162	↓	↓	↑	↓	↓	—
AN162(p5331)	—	↓	↓	↓	↓	↑
AN163	↓	—	↑	↑	↓	↑
AN163(p5341)	↓	—	—	↑	↑	↑

^a The changes in the enzyme activities and levels of quinones in the mutants are expressed relative to those of the wild type W3110 and are either higher than (↑), lower than (↓), or show no significant change from the wild type (—). The mutants containing cosmids are also defined relative to W3110.

assaying for the specific enzymes (Tables 7 and 8). Isomerase activity could be assayed and the results could be interpreted meaningfully, if precautions were taken, since the activity in wild-type cells was very low. The assay for PT activity was straightforward in the interpretation of the data, since the incorporation of the label was significantly above background. The HPT activity assay also showed high incorporation of label, but since the assay did not distinguish between *cis*- and *trans*-condensing activities it was more difficult to determine whether a mutation had occurred so as to affect these genes. Attempts were made to assay for the conversion of mevalonic acid to IPP (Fig. 1), but crude cell extracts gave no detectable incorporation (M. M. Sherman, unpublished data). None of the mutants was assayed for *ubiA* and *menA* gene product activity.

HPLC assays of the strains containing cosmids showed increases in the levels of Q-8 and UN-1 for all of the samples. It is difficult to assess the importance of the UN-1 peak until the second component is identified and its concentration relative to MK-8 is determined. UN-1 was linked to the level of Q-8 in several of the mutants, since there was a drastic alteration in the relative levels of Q-8 to those of UN-1. In particular, MS5015, MS5016, MS5031, MS5048, and AN163 all had at least a fourfold change in the Q-8/UN-1 ratio. It has been noted previously (20) that the ratio of Q-8 to MK-8 is

TABLE 8. Changes in the activities and levels of quinones in *ts* mutant strains^a

<i>ts</i> Mutant strain	Change in enzyme activity			Change in quinone level		
	Isomerase	PT	HPT	Q-8	UN-1	Q-8/UN-1
25	↓	↓	↓	—	↑	—
33	—	—	↓	↓	—	↓
38	↓	↓	—	—	↓	↑
51	—	↓	↓	↓	↓	↑
62	↓	↓	↓	↓	↓	—
101	—	↓	—	↓	ND	NA
112	↑	—	—	—	—	↑
301	—	↓	—	—	—	—

^a The *ts* mutant strains are those of Suzuki et al. (25). The changes in the enzyme activities and levels of quinones in the mutants are expressed relative to those of the parental strain PA3092 and are higher (↑) or lower (↓) or showed no significant change (—) from those in PA3092. Abbreviations: ND, Not detected; NA, not available.

variable in wild-type strains and is dependent on the age of the culture and other physiological factors. We attempted to minimize these effects by growing the strains to approximately equivalent cell densities. The other notable peak was Q-9, which was present at various levels in all of the complemented strains, as well as some of the *ts* mutants. This suggests that the enzyme, encoded for by *ubiA*, which is responsible for the prenylation of the aromatic nucleus, is not highly specific for the chain length of the polyprenyl diphosphate precursor.

Overall, we generated and characterized several mutants which showed alterations in the isoprene biosynthetic pathway. While there are similarities among these mutants with respect to phenotype, map position, enzymatic activity, or ubiquinone content, when all of the data are considered each mutant is unique. The strongest mutants were MS5015, MS5016, MS5031, MS5048, AN162, AN163, *ts* 33, *ts* 38, *ts* 51, and *ts* 101. Each presents distinct questions regarding isoprene biosynthesis and regulation of the pathway.

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