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Escherichia coli Open Reading Frame 696 Is *idi*, a Nonessential Gene Encoding Isopentenyl Diphosphate Isomerase

FREDERICK M. HAHN, ANTHONY P. HURLBURT,† AND C. DALE POULTER*

Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

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Isopentenyl diphosphate isomerase catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In eukaryotes, archaebacteria, and some bacteria, IPP is synthesized from acetyl coenzyme A by the mevalonate pathway. The subsequent isomerization of IPP to DMAPP activates the five-carbon isoprene unit for subsequent prenyl transfer reactions. In *Escherichia coli*, the isoprene unit is synthesized from pyruvate and glyceraldehyde-3-phosphate by the recently discovered nonmevalonate pathway. An open reading frame (ORF696) encoding a putative IPP isomerase was identified in the *E. coli* chromosome at 65.3 min. ORF696 was cloned into an expression vector; the 20.5 kDa recombinant protein was purified in three steps, and its identity as an IPP isomerase was established biochemically. The gene for IPP isomerase, *idi*, is not clustered with other known genes for enzymes in the isoprenoid pathway. *E. coli* FH12 was constructed by disruption of the chromosomal *idi* gene with the aminoglycoside 3′-phosphotransferase gene and complemented by the wild-type *idi* gene on plasmid pFMH33 with a temperature-sensitive origin of replication. FH12/pFMH33 was able to grow at the restrictive temperature of 44°C and FH12 lacking the plasmid grew on minimal medium, thereby establishing that *idi* is a nonessential gene. Although the V_{max} of the bacterial protein was 20-fold lower than that of its yeast counterpart, the catalytic efficiencies of the two enzymes were similar through a counterbalance in K_ms. The *E. coli* protein requires Mg^{2+} or Mn^{2+} for activity. The enzyme contains conserved cysteine and glutamate active-site residues found in other IPP isomerases.

Isoprenoid compounds are the most chemically diverse family of metabolites found in nature. Among their numerous essential functions, isoprenoids serve as structural components of membranes, mediate cellular redox chemistry, regulate reproductive cycles, serve as protective agents against damage by sunlight, relay messages during signal transduction, attract mates, defend against predators, and transport sugars during glycoprotein biosynthesis. Ubiquinone, dolichol, and prenylated tRNAs are examples of isoprenoids found in *Escherichia coli*. In eukaryotes, archaebacteria, and some bacteria, isopentenyl diphosphate (IPP) is the first intermediate in the pathway containing the characteristic isoprenoid 2-methylbutyl hydrocarbon unit (22). The carbon atoms in IPP are assembled from three molecules of acetyl coenzyme A by the classical mevalonate pathway. Following its synthesis, the carbon-carbon double bond in IPP is isomerized to create the potent electrophile dimethylallyl diphosphate (DMAPP), which then alkylates other molecules, including IPP, to ultimately form the numerous isoprenoid structures found in nature (21). A second pathway for biosynthesis of isoprenoids that does not involve mevalonate (the nonmevalonate pathway) was recently discovered in bacteria (23) and plant chloroplasts (16). In the nonmevalonate pathway, carbon atoms in the 2-methylbutyl unit are derived from pyruvate and glyceraldehyde phosphate. The three-carbon precursors are condensed with concomitant loss of carbon dioxide to produce D-deoxyxylulose phosphate (DXP) (26). The next step, formation of 2-methylerythritol-4-phosphate, involves the intramolecular rearrangement and reduction of DXP. The gene encoding DXP reductoisomerase, *yaeM*, was recently identified, and the encoded enzyme was characterized (29). The subsequent steps that lead to molecules with isoprenoid structures have not yet been defined, and at this point it is unclear whether IPP or DMAPP is synthesized first. In either case, an isopentenyl diphosphate: dimethylallyl diphosphate isomerase (IPP isomerase) would be required to interconvert the two compounds in order to provide IPP as a substrate for the chain elongation steps in the pathway and DMAPP as the initial reactive electrophile. Alternatively, IPP and DMAPP could be synthesized by independent routes or from a common intermediate that generates both molecules. In this scenario, there would not be an absolute requirement for IPP isomerase.

Many of the prenyl transfer reactions that occur after the isomerization of IPP to DMAPP are similar in organisms from all three domains, *Eucarya*, *Archaea*, and *Bacteria*. The most studied group of prenyltransferases is the one that catalyzes the short and medium-length chain elongation reactions. Phylogenetic correlations based on amino acid sequences clearly indicate that the proteins from *Eucarya*, *Archaea*, and *Bacteria* evolved from a common ancestor (3) despite different routes for the biosynthesis of IPP and DMAPP in these organisms. Much less is known about the phylogeny of IPP isomerase. The only bacterial enzymes that have been characterized are the *E. coli* protein described in this report and the *Rhodobacter capsulatus* IPP isomerase encoded by a gene located in its photosynthesis gene cluster (8). A putative *Mycobacterium tuberculosis* IPP isomerase was identified in database searches (National Center for Biotechnology Information [NCBI] accession no. Z95890). *E. coli* synthesizes isoprenoids via the nonmevalonate pathway (23), and it is likely that *M. tuberculosis* does as well since its genome does not contain known mevalonate pathway-related sequences. To date, the *R. capsulatus* genome has not been completely sequenced, and *R. capsulatus* has not been specifically reported as utilizing the nonmevalonate pathway. However, two other species from the alpha subdivision of *Bacteria*, *Rhodopseudomonas acidophila* and

* Corresponding author. Mailing address: Department of Chemistry, University of Utah, Salt Lake City, UT 84112. Phone: (801) 581-6685. Fax: (801) 581-4391. E-mail: poulter@chemistry.utah.edu.

† Present address: Ventana Genetics Inc., Salt Lake City, UT 84108.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli		
DH5α	Host for cloning vectors	Life Technologies
JM101	Host for disruption experiments and protein expression	Stratagene
FH12	JM101 *idi*::Kan^r	This work
Plasmids		
pCNTR	DNA cloning and shuttle vector; Ap^r	5′→3′
pAPHII294	pCNTR with 570-bp PCR product encoding IPP isomerase	This work
pARC306N	*E. coli* expression vector; Ap^r; *rec-7* hybrid promoter	29
pAPHIII22	pARC306N with 550-bp *Nde*I/*Xba*I fragment of pAPHII294	This work
pMAK705	Cm^r, temperature-sensitive origin of replication	S. R. Kushner
pUC4K	Contains 1.3-kb Kan^r cassette	Pharmacia
pAPHIII-*Bam*HI	pAPHIII22 with a unique *Bam*HI site inserted at the *Spe*I site of *idi*	This work
pAPHIII-*idi*::Kan^r	pAPHIII-*Bam*HI with the 1.3-kb Kan^r cassette from pUC4K inserted into *idi*	This work
pFMH33	pMAK705 with the 0.7-kb *Pvu*II-*Sph*I fragment containing wild-type *idi* from pAPHIII22	This work
pFMH35	pMAK705 with the 2.0-kb *Pvu*II-*Sph*I fragment containing *idi*::Kan^r from pAPHIII-*idi*::Kan^r	This work

[a] Ap, ampicillin; Kan, kanamycin; Cm, chloramphenicol.

Rhodopseudomonas palustris, are purported to rely on the nonmevalonate pathway (5).

Most of the amino acid sequences for IPP isomerases available from databases are for organisms which synthesize isoprenoids by the mevalonate pathway. A typical example is the enzyme from *Saccharomyces cerevisiae* (1). The yeast enzyme is a soluble monomer. Covalent modification of active site residues by mechanism-based inhibitors and subsequent generation of site-directed mutants of the reactive amino acids revealed that amino acids Cys139 and Glu207 are essential for catalysis (27). These residues were subsequently discovered in other IPP isomerases, including the recently reported IPP isomerase encoded by open reading frame 176 (ORF176) in the photosynthesis gene cluster of *R. capsulatus* (8). Although the sequence similarity to other IPP isomerases is relatively low, we now report that *E. coli* ORF696 (the previous designation in the *E. coli* genome database and now named b2889 [3a]; listed as ORF182 in NCBI BLAST searches), designated *idi*, is a nonessential gene that encodes IPP isomerase.

MATERIALS AND METHODS

Materials. [1-^{14}C]IPP was purchased from DuPont NEN. Nalidixic acid was from Sigma. DE52 ion-exchange resin was from Whatman. All restriction endonucleases were from GIBCO-BRL. T4 DNA ligase was from Boehringer Mannheim. AmpliTaq polymerase was from Perkin-Elmer. Plasmid pMAK705 was provided by S. R. Kushner (University of Georgia, Athens). Synthesis of oligonucleotides and DNA sequencing were conducted by the Protein/DNA Core Facility at the Utah Regional Cancer Center.

General procedures. Minipreparations of plasmid DNA for restriction analysis were obtained by the boiling method as described by Sambrook et al. (24). Large-scale plasmid preparations (>100 μg) were performed with the purification kit from Qiagen. DNA fragments were purified on agarose gels (IBI) with a Geneclean II kit from Bio 101. Restriction digestions, ligations, and *E. coli* transformations were conducted as described by Sambrook et al. (24).

IPP isomerase was assayed by the acid lability procedure (25). Chromatography on DE52 and phenyl-Sepharose was conducted at 4°C, and chromatography on POROS-HQ was conducted on ice. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the discontinuous system of Laemmli (13), and the gels were stained with Coomassie brilliant blue R (Sigma). Protein concentrations were determined by the method of Bradford (2), with bovine serum albumin as a standard.

Strains and plasmids. All strains and plasmids used in this study are listed in Table 1. Growth conditions for *E. coli* DH5α and JM101 were described previously (9). *E. coli* FH12 was grown at 30 or 44°C in Luria broth (LB) or M9 minimal medium (24) supplemented with thiamine hydrochloride (0.0002%, wt/vol) and Casamino Acids (1%, wt/vol). The antibiotics chloramphenicol (Cam) (30 μg/ml) and/or kanamycin (Kan) (25 μg/ml) were used as necessary.

DNA amplification and cloning. *E. coli* K-12 genomic DNA was digested with *Nde*I and amplified by PCR using DNA primers AH1 (5′-GGAATTCCAT*ATG* CAAACGGAACACGTCATTTTAT-3′) and AH2 (5′-GCAGATCTTTATTTA AGCTGGGTAAATGCA G-3′). Primer AH1 (upstream) included adjacent *Eco*RI and *Nde*I sites (underlined), which incorporate the start codon (italics) for ORF696. Primer AH2 (downstream) contained an *Xba*I site (underlined). The ~570-bp product was size fractionated by agarose gel electrophoresis and purified by using a Geneclean II kit. The PCR product was then cloned into pCNTR by blunt-end ligation, following the protocol for the General Contractor DNA cloning system (5′→3′), to yield pAPHII294 and sequenced.

Plasmid pFMH35. A temperature-sensitive pMAK705 derivative containing the disrupted *idi* gene was constructed as follows. Plasmid pAPHIII22 (see below) was restricted with *Spe*I, a unique site contained in *idi*, and the resultant 5′ overhangs were partially filled in by incubation with Klenow enzyme, dTTP, and dCTP. A synthetic oligonucleotide (5′-AGGGATCC-3′) was self-annealed to create a double-stranded DNA fragment with two-base overhangs. This DNA fragment, containing the restriction site *Bam*HI, was inserted into *idi* to give pAPHIII-*Bam*HI. Restriction of pAPHIII-*Bam*HI with *Bam*HI was followed by a partial fill-in of the resultant overhangs with Klenow enzyme, dGTP, and dATP. Restriction of pUC4K with *Sal*I yielded a 1.3-kb fragment containing the aminoglycoside 3′-phosphotransferase gene that was subsequently treated with Klenow enzyme, dTTP, and dCTP to create compatible sticky ends for ligation into pAPHIII-*Bam*HI. The ligation product was restricted with *Pvu*II and *Sph*I to give a 2-kb DNA fragment containing *idi* disrupted with the gene imparting kanamycin resistance (Kan^r). The *Pvu*II-*Sph*I fragment was then ligated into the *Hin*dII and *Sph*I sites of pMAK705 to create pFMH35, the delivery vehicle for gene disruption of chromosomal *idi*.

Construction of *E. coli* FH12. An *E. coli* strain containing the chromosomal *idi* gene disruption was constructed as described by Hamilton et al. (11). *E. coli* JM101 competent cells were transformed with pFMH35 and grown at 30°C to an optical density at 600 nm of ~0.6 in LB containing chloramphenicol (LB/Cam). Approximately 10,000 of the transformed cells were then plated on LB/Cam that was prewarmed to 44°C. Following incubation of the plates at 44°C, several of the colonies were used to individually inoculate 3 ml of LB/Cam and grown at 44°C until confluent. Two 100-ml LB/Cam cultures were started with 0.5 ml from two of the 4-ml cultures, chosen at random, and grown overnight at 30°C. Samples from the two cultures were serially diluted 5×10^5-fold, and 5 μl was plated on LB/Cam. Following incubation at 30°C, the resultant colonies were used to inoculate LB/CAM and LB containing kanamycin (LB/Kan) for plasmid DNA isolation and restriction analysis. The process of inoculating the 100 ml of LB/Cam cultures with 0.5 ml of the previous culture and determining the identity of plasmids isolated from cells corresponding to each culture was repeated five times. Restriction analysis of plasmid DNA isolated from cultures started with three colonies derived from the fifth outgrowth showed a DNA fragment pattern identical to that of restricted pFMH33, a pMAK705 derivative containing just the wild-type *idi* (wt *idi*) gene, thus indicating that the chromosomal gene disruption had occurred. A portion of each of the cultures corresponding to the three identified colonies, FH12/pFMH33-(6), -(9), and -(11), was used to inoculate 100 ml of LB/Cam/Kan for large-scale plasmid isolation for sequence analysis.

Growth of *E. coli* FH12/pFMH33 at 30°C and at 44°C. Three 50-ml samples of LB/Kan were inoculated with 0.5 ml from each of the LB/Cam/Kan cultures corresponding to FH12/pFMH33-(6), -(9), and -(11) and grown overnight at 30°C. The cultures were then diluted 10,000-fold, and 20 μl was spread on LB/Kan plates that were prewarmed to 44°C or were at room temperature. The set of prewarmed plates were incubated overnight at 44°C, and the room temperature plates were incubated at 30°C overnight.

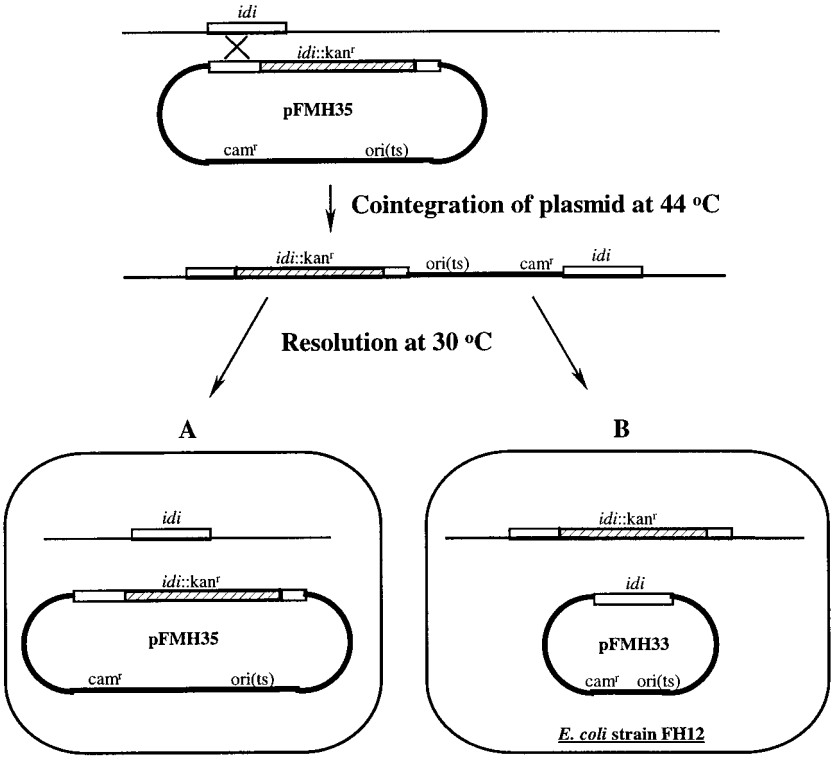

FIG. 1. Construction of *E. coli* FH12 containing the disrupted chromosomal *idi* gene. Plasmid pFMH35 (pMAK705-*idi*::Kan^r), a pMAK705 derivative containing a temperature-sensitive origin of replication [ori(ts)], was integrated into the chromosome of its host via homologous recombination when grown in the presence of chloramphenicol (cam) at the nonpermissive temperature of 44°C. A second event, resolution of a plasmid from the cointegrated *E. coli* genome, was selected by growth at the permissive temperature of 30°C. Two variants were excised as illustrated. The resolved plasmid (A) resembles the original gene disruption vehicle pFMH35, denoting that wt *idi* remained in the chromosome; the excised plasmid (B) was identical to pFMH33 (pMAK705-wt *idi*) containing wt *idi*, establishing that the chromosomal *idi*::Kan^r gene disruption had taken place.

Loss of episomal *idi* from strain FH12/pFMH33. Colonies resulting from the growth of *E. coli* FH12/pFMH33 at 44°C were grown in LB/Kan to stationary phase at 44°C. A portion of each culture was streaked on both LB/Kan and LB/Cam media, and the plates were incubated overnight at 30°C to test for the presence of the Cam^r marker. Genomic DNA was isolated from cultures corresponding to cells exhibiting chloramphenicol, but not kanamycin, sensitivity by using an Easy-DNA kit (Invitrogen) according to the manufacturer's instructions. PCR amplification of the isolated DNA was performed with the primers FH1208-1 (5′-GCTCTAGAATGCAAACGGAACACGTCATTTTATTGAATGC-3′), FH1207-2 (5′-CGGAATTCTTATTTAAGCTGGGTAAATGCAGATAATCG-3′), and FH1207-3 (5′-GCTCTAGAATGAGCCATATTCAACGGGAAACGTCTTGC-3′).

Growth of *E. coli* FH12 on minimal medium. FH12 was grown to stationary phase at 30°C in LB/Kan medium. Following a 10,000-fold dilution of the culture with M9 minimal medium, a 20-μl aliquot was plated on M9 minimal medium containing Casamino Acids and vitamin B_1 and incubated overnight at 30°C.

Expression of *E. coli* IPP isomerase by *E. coli*. Restriction of pAPHII294 with *Nde*I and *Xba*I yielded a 550-bp fragment which contained the *E. coli* ORF696. The *Nde*I/*Xba*I fragment was cloned into the *E. coli* expression vector pARC306N (28). The resulting plasmid, pAPHIII22, was used to transform *E. coli* JM101. Protein synthesis was induced with nalidixic acid as previously described (9).

Purification of *E. coli* IPP isomerase. Four grams of cell paste from JM101/pAPHIII22 was suspended in 20 ml of 5 mM potassium phosphate (KP) buffer (pH 7.5) containing 10 mM β-mercaptoethanol (BME) and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication. After centrifugation at 23,700 × *g* for 20 min, the resulting supernatant was loaded on a DE52-cellulose column (1.5 by 25 cm) previously equilibrated with 5 mM KP–10 mM BME (pH 7.5). The column was eluted at 2 ml/min with a linear gradient of buffer A to 75% 500 mM KP (pH 7.5)–10 mM BME over 200 ml. Fractions containing IPP isomerase activity were collected and combined. The combined fractions were titrated to 1.0 M $(NH_4)_2SO_4$ with stirring at 4°C, using 2 M $(NH_4)_2SO_4$–10 mM BME (pH 7.0). The solution was loaded on a phenyl-Sepharose column (1.5 by 20 cm) preequilibrated with 1.0 M $(NH_4)_2SO_4$–10 mM BME (pH 7.0). The column was eluted with a linear gradient to 100% 10 mM BME over 100 ml. Active fractions were combined, and 1 M Tris (pH 7.0) was added to a final concentration of 20 mM Tris. The resulting solution was then loaded onto a 4.6-by 100-mm POROS-HQ (Perseptive Biosystem) column preequilibrated with 20 mM Tris–1 mM dithiothreitol (DTT; pH 7.0). The column was eluted with a linear gradient to 50% 20 mM Tris–1 M NaCl–1 mM DTT, pH 7.0) over 55 ml. Fractions were assayed for IPP isomerase activity and protein content after each purification step. Purified protein was stored at −20°C in buffer containing 20% glycerol until used.

Metal ion dependence. The effects of Mg^{2+} and Mn^{2+} on V_{max} were measured in 50 mM HEPES (pH 7.0)–0.8 mM DTT–200 mM KCl–250 μM [1-^{14}C]IPP (2 μCi/μmol). Stock solutions of substrate were diluted with buffer containing the metal ion prior to the initiation of the isomerization reaction with enzyme. All assays were performed in duplicate at 37°C.

Kinetic parameters. K_m and V_{max} for IPP were determined by varying IPP concentrations between 1.6 and 80 μM [1-^{14}C]IPP (5 μCi/μmol). Assays of the initial velocities were performed in triplicate at 37°C in buffer containing 50 mM HEPES (pH 7.0), 200 mM KCl, 20 mM $MgCl_2$, 0.8 mM DTT, and purified recombinant *E. coli* IPP isomerase (0.83 μM). Conversion of IPP to DMAPP was limited to 10% or less. K_m and V_{max} for IPP were obtained by using Grafit (14).

K_ms for IPP and DMAPP were determined simultaneously from product inhibition profiles for DMAPP. Reactions were run at 37°C in 50 mM HEPES (pH 7.0)–200 mM KCl–20 mM $MgCl_2$–0.8 mM DTT–0.83 nM recombinant *E. coli* IPP isomerase. K_ms for IPP and DMAPP were obtained by fitting with equations for competitive inhibition by using Grafit.

RESULTS

***E. coli idi* is a nonessential gene.** Plasmid pFMH35, a pMAK705 derivative, was constructed to disrupt the *E. coli idi* gene encoded by ORF696. The use of pMAK derivatives as vehicles for creating chromosomal disruptions in specific *E. coli* genes via homologous recombination is described by Hamilton et al. (11) as illustrated for *idi* in Fig. 1. Cells resulting from the transformation of *E. coli* JM101 with pFMH35 were grown at both 30 and 44°C. Growth at 44°C in the presence of chloramphenicol forced integration of pFMH35 into the *E. coli* genome. The frequency of the cointegration event was determined to be 5×10^{-4} by dividing the number of cells surviving

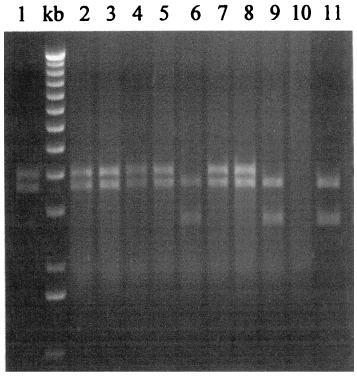

FIG. 2. Agarose gel of isolated plasmid DNA restricted with *Nde*I. *E. coli* cells corresponding to lanes 6 and 9 have undergone resolution of a plasmid containing wt *idi* resulting in the incorporation of *idi*::Kan^r in the chromosome. Lanes: 1, pFMH35; kb, 1-kb DNA ladder (GIBCO-BRL); 2 to 9, plasmid DNA isolated from cells following five outgrowths of the cointegrated strain; 10, plasmid miniprep from the cointegrated strain; 11, pFMH33.

at 44°C by the number of those viable from an equivalent sample at 30°C. Following the propagation of several of the cointegrants at 44°C, a series of outgrowths were performed in LB/Cam/Kan by seeding cultures with a portion of the previous culture and allowing growth at 30°C to stationary phase.

Restriction analysis was performed on plasmid DNA isolated from colonies corresponding to each outgrowth. As shown in Fig. 2, *Nde*I digestion of plasmid DNA corresponding to a small percentage of cells from the fifth outgrowth revealed a restriction pattern identical to that of *Nde*I-restricted pFMH33, a pMAK705 derivative containing only the *idi* gene. This finding indicated that resolution of the wt *idi* gene from the cointegrated genome had taken place in those cells, thereby depositing the *idi*::Kan^r disruption in the chromosome. The opposite scenario, in which the disrupted *idi* gene was excised from the genome to give a plasmid identical to pFMH35, was evident in 13 of 16 instances. Large-scale cultures of strains FH12/pFMH33-(6), -(9), and -(11) containing the desired gene disruption were grown, and the plasmid DNA was isolated for sequence analysis. The three resolved plasmids were identical to pFMH33 synthesized independently.

To determine if *idi* is an essential gene, cultures of FH12/pFMH33-(6), -(9), and -(11) were diluted 10,000-fold, and 20-μl aliquots were spread on LB/Kan plates that were prewarmed to 44°C. The colonies grew at both 30 and 44°C, thus establishing that disruption of *idi*, encoded by ORF696, was not lethal. To further characterize the *idi* disruption, small-scale LB/Kan cultures were started with eight colonies from the 44°C, FH12/pFMH33-(11) plate. Plasmid DNA was isolated from the cultures grown at 44°C, and agarose gel electrophoresis indicated that no plasmid DNA was present in any of the samples. To confirm the loss of episomal *idi* from the eight 44°C cultures, a portion of each was streaked on both LB/Kan and LB/Cam and grown at 30°C. All of the FH12 isolates were chloramphenicol sensitive, confirming the absence of the Cam^r marker. Genomic DNA was isolated from FH12-(11) cells grown at 44°C and analyzed by PCR to determine if only the *idi*::Kan^r disruption was present. Two sets of primers were used. One set (FH1208-1 and FH1207-2) corresponded to the *idi* gene, and the second (FH1207-2 and FH1207-3) corresponded to both the *idi* gene and the Kan^r marker. The lack of a chromosomal copy of wt *idi* was confirmed by the absence of PCR products corresponding to the wt *idi* gene. FH12 cells missing the wt *idi* gene encoded by ORF696 were also able to grow on M9 minimal medium supplemented only with Casamino Acids and vitamin B_1. Thus, the nonessential nature of *idi* was established.

Overproduction and purification of *E. coli* IPP isomerase. With *E. coli* K-12 genomic DNA as a template, DNA containing ORF696 flanked by 5′ *Nde*I and 3′ *Xba*I restriction sites was synthesized by PCR using primers AH1 and AH2. The product was purified by Geneclean and inserted into pCNTR to give pAPHII294. The 550-bp *Nde*I/*Xba*I fragment containing ORF696 was cloned into *E. coli* expression vector pARC306N to give plasmid pAPHIII22. Cultures of JM101/pAPHIII22 were grown at 37°C in supplemented M9 minimal medium, and plasmid-directed synthesis was induced by addition of nalidixic acid. The low background activity of native *E. coli* IPP isomerase in *E. coli* JM101/pARC306N was not detected by the standard assay.

Recombinant *E. coli* IPP isomerase was purified from cell extract in three steps by ion-exchange chromatography on DE52-cellulose, hydrophobic interaction chromatography on phenyl-Sepharose, and ion-exchange chromatography on a POROS-HQ column. This procedure gave enzyme that was greater than 97% pure as judged by SDS-PAGE. SDS-PAGE of the supernatant and pellet obtained by low-speed centrifugation of disrupted cells showed a strong band in the supernatant at 24,500 Da, which corresponded to the single band seen for purified protein. The predicted mass of the gene product of ORF696 is 20,500 Da. While this discrepancy is somewhat larger than often encountered, a similar trend was seen for yeast IPP isomerase (1).

Kinetic properties of recombinant *E. coli* IPP isomerase. The recombinant *E. coli* enzyme, like other IPP isomerases, requires Mg^{2+} or Mn^{2+}. Mg^{2+} concentrations between 10 μM and 230 mM were tested, and the activity of the protein increased with increasing Mg^{2+} concentration to a maximal value at 20 mM Mg^{2+}. The effects of Mn^{2+} concentration were tested from 1 μM to 10 mM. Isomerase activity increased to a maximal value at 100 μM Mn^{2+}. However, unlike activities of other isomerases, the activity of the *E. coli* enzyme reached a plateau at higher Mn^{2+} concentrations rather than decreasing sharply.

A plot of the initial velocity for formation of DMAPP versus the concentration of IPP was hyperbolic, with a K_m for IPP of 7.9 ± 0.1 μM. This value is similar to that reported for an impure preparation of *E. coli* isomerase (7) and somewhat lower than the value of 43 μM obtained for homogeneous *S. cerevisiae* enzyme (28). The V_{max}, 0.97 ± 0.01 μmol min^{-1} mg^{-1}, for recombinant *E. coli* IPP isomerase is 20-fold less than the value reported for the *S. cerevisiae* enzyme (28). While there is no convenient assay to monitor the IPP isomerase reaction in the reverse direction, the K_m for DMAPP can be readily determined from product inhibition studies of the initial velocity in the forward reaction (20). As seen in Fig. 3, a plot of $1/v$ versus 1/IPP concentration at different fixed concentrations of DMAPP gave a graph consistent with competitive inhibition. The K_ms for DMAPP and IPP obtained by fitting the data were 14.3 ± 0.8 and 9.5 ± 0.5 μM, respectively.

DISCUSSION

The mevalonate and nonmevalonate pathways converge at the fundamental five-carbon building blocks IPP and DMAPP. In the mevalonate pathway, IPP isomerase activity is required to convert IPP to DMAPP, the electrophilic isoprenoid unit required for subsequent prenyl transfer reactions. In *S. cerevisiae*, IPP isomerase is encoded by *IDI1*, an essential single-copy gene (19). The gene has also been identified in several other eukaryotes, including other fungi, mammals, and plants (22).

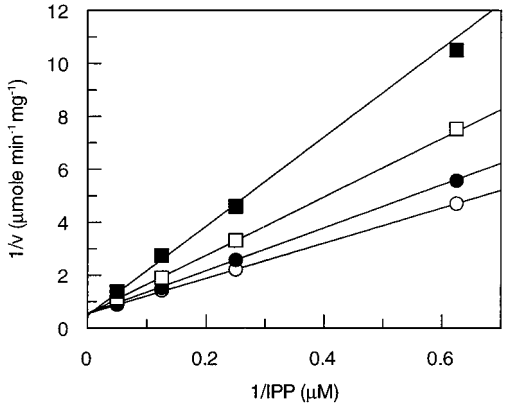

FIG. 3. Determination of Michaelis constants for IPP and DMAPP by product inhibition kinetics. Shown is a double-reciprocal plot of initial velocity versus IPP concentration at fixed DMAPP concentrations. IPP concentrations were 1.6, 4, 8, and 20 μM; DMAPP concentrations were 4 (open circles), 8 (closed circles), 16 (open squares), and 32 (closed squares) μM. All assays were performed at 37°C in 50 mM HEPES buffer (pH 7.0) containing 200 mM KCl, 20 mM $MgCl_2$, and 0.8 mM DTT.

The nonmevalonate pathway has not yet been fully characterized. Labeling experiments in bacteria and plants have established that the carbon atoms in the isoprene units are derived from DXP, which is synthesized from pyruvate and D-glyceraldehyde phosphate (4). Genes encoding DXP synthase (*dxs*) have been found in *E. coli* and plants (4). Recently, the gene for a DXP reductoisomerase (*yaeM*) was discovered in *E. coli* (29). The encoded protein converts DXP to methylerythritol phosphate. This appears to be the first pathway-specific reaction in the nonmevalonate pathway, although efficient incorporation of methylerythritol phosphate into isoprenoids has not yet been demonstrated. The remaining steps between methylerythritol phosphate and IPP are still unknown.

The gene for *E. coli* IPP isomerase, *idi*, is located at 65.3 min on the chromosome between ORF505, whose encoded protein has strong similarity to *Bacillus subtilis* xanthine permease, and the lysyl-tRNA synthetase gene (*lysS*). *yaeM*, the gene that encodes DXP reductoisomerase, is located at 4.2 min (29). Farnesyl diphosphate (FPP) synthase, the enzyme that follows isomerase in the isoprenoid pathway, is encoded by *ispA* (6). *ispA* is located at 9.5 min on the *E. coli* chromosome and is cotranscribed with *dxs* and *yajO*, an ORF encoding a putative NAD(P)H-dependent reductase (18). Thus, *idi* is not clustered with other genes in the isoprenoid pathway. *idi* is not essential in *E. coli*. FH12/pFMH33, in which the chromosomal copy of *idi* was disabled by insertion of the aminoglycoside 3′-phosphotransferase gene, was viable at the restrictive temperature for replication of the plasmid containing an episomal copy of wt *idi*. In addition, FH12 lacking pFMH33 grew on minimal medium. We also found that the copy of *idi* located in the photosynthesis gene cluster of *R. capsulatus* is not essential (8). In contrast, the genes encoding DXP reductoisomerase (*yaeM*) and FPP synthase (*ispA*) are single copy and essential (6, 29). Given that FPP synthase activity is required in *E. coli*, strain FH12 must be capable of synthesizing both IPP and DMAPP. If the bacterium possesses a second IPP isomerase, its amino acid sequence is sufficiently different from that of the *idi* gene product that it was not identified in our searches of databases for the *E. coli* genome. Alternatively, the nonmevalonate pathway may produce both IPP and DMAPP from a common precursor. In this event, IPP isomerase might not be required but would permit the bacterium to optimize utilization of the two intermediates. Two *IDI* genes have been found in the genome of the plant *Arabidopsis thaliana* (15). Higher plants appear to contain a cytosolic mevalonate pathway and a chloroplastic nonmevalonate pathway for biosynthesis of isoprenoid compounds (4). The two *A. thaliana IDI* genes encode proteins that may contain amino-terminal chloroplastic targeting sequences. At this point it is unclear if either enzyme participates in the nonmevalonate pathway found in the chloroplast. However, database searches using identified IPP isomerases as the query sequence have failed to identify putative IPP isomerases in several organisms whose genomes have been completely sequenced, including *B. subtilis*, *Haemophilus influenzae*, *Aquifex aeolicus*, *Borrelia burgdorferi*, and *Methanobacterium thermoautotrophicum*.

Kinetic studies have been reported for several IPP isomerases. All of the enzymes require a divalent metal, either Mg^{2+} or Mn^{2+}, for turnover. The eukaryotic enzymes are substantially more active catalysts than *E. coli* isomerase, with k_{cat}s of ~1.8 to 11 s^{-1} for the eukaryotic enzymes (1, 10), versus 0.33 s^{-1} for the *E. coli* protein. However, all of the enzymes have similar catalytic efficiencies (k_{cat}/K_m) because the Michaelis constants of the bacterial isomerases are lower than those of their eukaryotic counterparts. The k_{cat}/K_m of 4.2×10^4 M s^{-1} for *E. coli* isomerase is substantially below the diffusion controlled rate for addition of substrates typically associated with enzymes under pressure to perform at an optimal level (12). Similar catalytic efficiencies for the other isomerases suggest that the interconversion of IPP and DMAPP is not normally a rate-limiting reaction in isoprenoid metabolism.

Eukaryotic IPP isomerases from fungal (1, 9), plant (15), and animal (10) sources have a conserved cysteine residue (boldface) in a TNTC**C**SHPL motif and a conserved glutamate residue in a WGEHEx**E**Y motif. These amino acids correspond to C139 and E207 in the *S. cerevisiae* enzyme (1). The sulfhydryl moiety of C139 is readily alkylated by a variety of active-site-directed irreversible inhibitors and was shown to be essential for catalysis by site-directed mutagenesis experiments (27). The conserved glutamate in the wild-type protein was not prone to alkylation. However, when the nucleophilic sulfhydryl group was replaced by a less reactive hydroxyl in the C139S mutant, incubation with the same set of inhibitors that modified resulted in alkylation of the side chain carboxylate of E209. Site-directed mutagenesis experiments demonstrated that the conserved glutamate is also required for isomerase activity.

The active-site cysteine and glutamate residues are also found in the bacterial IPP isomerases from *E. coli*, *R. capsulatus* (8), and the translated region of an unidentified ORF in *M. tuberculosis* (NCBI accession no. Z95890) that encodes a protein with substantial similarity to the *E. coli* enzyme. *E. coli* isomerase shares approximately 30% identity with its eukaryotic counterparts at the amino acid level. Interestingly, there is only 27% identity between the *E. coli* and *R. capsulatus* IPP isomerases, although both species are classified as purple bacteria. There is, in addition, more variation among the amino acids in the regions surrounding the conserved cysteine and glutamate residues in the bacterial proteins than seen for their eukaryotic counterparts. Thus, the overall consensus sequences for the regions containing the active-site Cys and Glu residues (boldface) are reduced to Nxx**C**xHP and Ex**E** when genes from members of both *Bacteria* and *Eucarya* are included. In *E. coli* isomerase, the cysteine residue corresponding to C138 in the *S. cerevisiae* protein is replaced by valine. That residue was modified along with C139 during the labeling experiments with the yeast enzyme (27). Although the amino acid is conserved in all other isomerases characterized to date, the C138A mutant of yeast isomerase showed only a slight

reduction in catalytic activity relative to the wild-type protein, demonstrating that the amino acid is not essential (27).

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