

Identification of an Archaeal Type II Isopentenyl Diphosphate Isomerase in *Methanothermobacter thermautotrophicus*

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Isopentenyl diphosphate (IPP):dimethylallyl diphosphate isomerase catalyzes the interconversion of the fundamental five-carbon homoallylic and allylic diphosphate building blocks required for biosynthesis of isoprenoid compounds. Two different isomerases have been reported. The type I enzyme, first characterized in the late 1950s, is widely distributed in eukaryota and eubacteria. The type II enzyme was recently discovered in *Streptomyces* sp. strain CL190. Open reading frame 48 (ORF48) in the archaeon *Methanothermobacter thermautotrophicus* encodes a putative type II IPP isomerase. A plasmid-encoded copy of the ORF complemented IPP isomerase activity in vivo in *Salmonella enterica* serovar Typhimurium strain RMC29, which contains chromosomal knockouts in the genes for type I IPP isomerase (*idi*) and 1-deoxy-D-xylulose 5-phosphate (*dxs*). The *dxs* gene was interrupted with a synthetic operon containing the *Saccharomyces cerevisiae* genes *erg8*, *erg12*, and *erg19* allowing for the conversion of mevalonic acid to IPP by the mevalonate pathway. His₆-tagged *M. thermautotrophicus* type II IPP isomerase was produced in *Escherichia coli* and purified by Ni²⁺ chromatography. The purified protein was characterized by matrix-assisted laser desorption ionization mass spectrometry. The enzyme has optimal activity at 70°C and pH 6.5. NADPH, flavin mononucleotide, and Mg²⁺ are required cofactors. The steady-state kinetic constants for the archaeal type II IPP isomerase from *M. thermautotrophicus* are as follows: K_m , 64 μM ; specific activity, 0.476 $\mu\text{mol mg}^{-1} \text{min}^{-1}$; and k_{cat} , 1.6 s^{-1} .

The isoprenoid biosynthetic pathway is ubiquitous across all three kingdoms of life. Over 36,000 natural products derived from the fundamental five-carbon isoprenoid building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) have now been identified (3). Many of these molecules have important biological functions, including glycoprotein synthesis (dolichols), inter- and intracellular signaling (prenylated proteins and steroidal hormones), membrane structure (steroids), electron carriers during redox reactions (ubiquinones), photoprotection (carotenoids), photosynthesis (chlorophyll), and defense against predators (sesquiterpenes and pyrethrins).

There are two independent biosynthetic routes to IPP and DMAPP (Fig. 1). In the mevalonate (MVA) pathway, first discovered in the late 1950s (4, 6), IPP is synthesized from mevalonic acid by the consecutive action of mevalonate kinase (MVA kinase), phosphomevalonate kinase (PMVA kinase), and mevalonate diphosphate decarboxylase (DPMVA decarboxylase). The isomerization of IPP to DMAPP is a mandatory step needed to create the electrophilic allylic diphosphates needed for subsequent prenyl transfer reactions. IPP isomerase is an essential enzyme in archaea, eukaryota, and some gram-positive eubacteria, where IPP is synthesized by the MVA pathway. More recently, it was discovered that IPP and DMAPP are synthesized from D-glyceraldehyde phosphate and pyruvate by the methyl erythritol phosphate (MEP) pathway found in many eubacteria, cyanobacteria, and plant chloroplasts (18, 28). In the final step of the MEP pathway, the *ispH*

gene product synthesizes IPP and DMAPP from 4-hydroxydimethylallyl diphosphate (Fig. 1) (26). In organisms that utilize the MEP pathway, *idi* is not essential or does not exist (10).

In 1997, *Methanothermobacter thermautotrophicus* was the first archaeon to have its genome fully sequenced (34). At that time there were no annotations for an open reading frame (ORF) similar to the *idi* genes previously identified in eukaryota and eubacteria, although Zhang and Poulter had reported activity for IPP isomerase in cell extracts from *M. thermautotrophicus* (47). Recently, Kaneda et al. identified an unclassified ORF in the MVA pathway gene cluster of *Streptomyces* sp. strain CL190 that encoded a new form of IPP isomerase (16). All members of the domain *Archaea* sequenced so far have ORFs for the type II enzyme. In addition, the type II isomerase is found in many pathogenic gram-positive eubacteria, cyanobacteria, and obligate parasitic eubacteria. The type I and type II isomerases have different structures and different cofactor requirements, suggesting that they catalyze isomerizations by different chemical mechanisms. We now report that an *idi* deletion in a mevalonate-dependent strain of *Salmonella enterica* serovar Typhimurium is complemented by the putative *idi* gene from *M. thermautotrophicus* and biochemical characterization of the encoded type II IPP isomerase.

MATERIALS AND METHODS

Materials. Genomic DNA from *M. thermautotrophicus* was isolated from frozen cells, provided by Lacy Daniels (University of Iowa), using a genomic DNA isolation kit from Qiagen. [1-¹⁴C]isopentenyl diphosphate was purchased from Amersham. L-Arabinose, flavin mononucleotide (FMN), reduced form of NADP (NADPH), and mevalonic acid were purchased from Sigma. Imidazole was purchased from Acros Organics. Ni-nitrilotriacetic acid (NTA) agarose resin was purchased from Qiagen. All restriction endonucleases, Klenow fragment, and T7 DNA polymerase were purchased from New England Biolabs. *Taq* DNA polymerase was purchased from Promega. Deoxynucleoside triphosphates and T4

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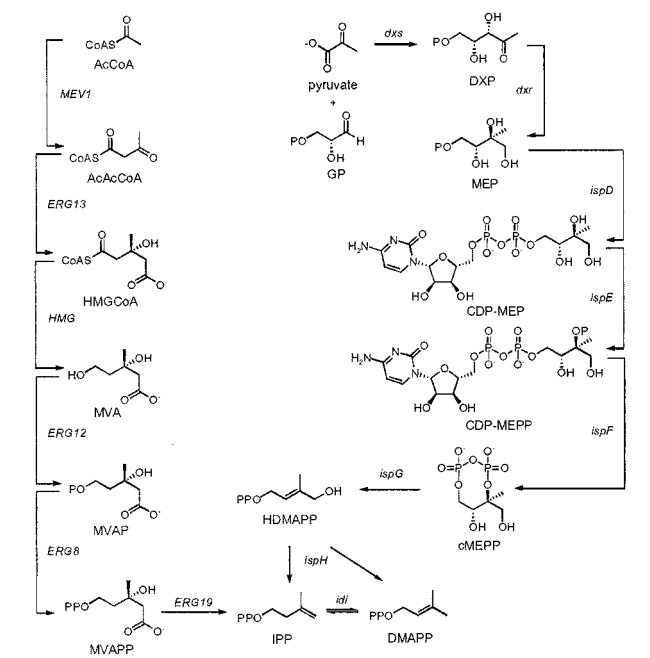


FIG. 1. MVA (left) and MEP (right) biosynthetic pathways to IPP and DMAPP. Abbreviations: AcCoA, acetyl CoA; AcAcCoA, acetoacetyl-CoA; HMGCoA, 3-hydroxy-3-methylglutaryl-CoA; MVAP, phosphomevalonate; MVAPP, diphosphomevalonate; DXP, deoxyxylulose phosphate; GP, glyceraldehyde phosphate; MEP, methylerythritol phosphate; CDP-MEP, cytidine methylerythritol diphosphate; COP-MEPP, cytidine phosphomethylerythritol diphosphate; cMEPP, cyclomethylerythritol diphosphate; HDMAPP, hydroxydimethylallyl diphosphate.

DNA ligase were purchased from Pharmacia. pBADA Myc/His and *Escherichia coli* BL21 DE3/pLysE were purchased from Invitrogen. *S. enterica* serovar Typhimurium shuttle strain TR6579 was provided by J. R. Roth (University of Utah, Salt Lake City, Utah). Oligonucleotide primers were synthesized by the

Protein/DNA Core Facility of the Utah Regional Cancer Center. 2-C-Methyl-D-erythritol was synthesized by the procedure of Duvold et al. (7).

General methods. Minipreparations of plasmid DNA for restriction analysis were obtained by using a Qiagen plasmid miniprep kit. DNA fragments were purified in agarose gels (Bio-Rad) using a gel purification kit from Qiagen. Restriction digestion, ligation, and transformation of competent *E. coli* and *S. enterica* serovar Typhimurium cells were conducted as described by Sambrook et al. (29) PCR was performed using the polymerase mix provided in the Advantage PCR kit from Clontech. Radioactivity was measured in Cytosint scintillation fluid (ICN Biomedicals). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system of Laemmli (19). The gels were stained with Coomassie brilliant blue R from Sigma. In-gel tryptic digestion was performed by the procedure of Hellman et al. (13). DNA was sequenced at the Health Sciences Center Sequencing Facility, Eccles Institute of Human Genetics, University of Utah.

Bacterial strains and growth conditions. All *S. enterica* serovar Typhimurium strains are derived from *S. enterica* serovar Typhimurium strain LT2. A high-frequency transducing mutant of phage P22 (HT105 *int*) was used to mediate all transductional crosses (31). Procedures for propagation of phage and transductional crosses have been described previously (2). The bacterial strains used in this study are listed in Table 1 and were grown at 37°C in Luria-Bertani (LB) medium supplemented with the following antibiotics and concentrations as necessary: ampicillin (AMP), 50 µg/ml; chloramphenicol (CAM), 34 µg/ml; and tetracycline (TET), 25 µg/ml. When needed, 0.2% L-arabinose (AR), 5 mM mevalonic acid (MEV), 0.3 mg of 2-C-methyl-D-erythritol (ME) per ml, and 3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were used.

Sequence analysis. Homology searches were performed at the National Center for Biotechnology Information (NCBI) website. Pairwise sequence alignments were performed with Vector NTI (Informax) using the Clustal W (1.74) algorithm.

Plasmid constructions. The plasmids used in this study are listed in Table 1. PCR primers were designed incorporating a 5' *Bgl*II site (underlined) and a 3' *Hind*III site (underlined) complementary to ORF48 from *M. thermotrophicus* cDNA as follows: *Bgl*II 5'-AGATCTATTATTCGGATAGGAACTGGAG C-3' and *Hind*III 5'-AAGCTTTAATGACCTCTGGCGTATTTTTT-3'. The native Met start codon was mutated to Ile (bold), and the native stop codon was mutated to Leu (bold). The gel-purified 1.1-kb PCR product was A-tailed and ligated into the subcloning vector pGEM-T Easy (Promega) to give pJMSB0335. pJMSB0335 was sequenced to verify that the 1.1-kb insert was identical to the deposited sequence for *M. thermotrophicus* ORF48. pJMSB0335 was restriction digested with *Bgl*II and *Hind*III and ligated into the doubly digested expression vector pBAD Myc/His A (Invitrogen) to give the expression plasmid pJMSB0338 containing a C-terminal His₆ tag.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source
<i>E. coli</i>		
DH5α	Host for cloning vectors	Life Technologies
BL21 DE3/pLysE	Host for protein synthesis, Cam ^r	Novagen
JMSB0338	BL21 DE3/pLysE containing pJMSB0338; Amp ^r Cam ^r	This work
<i>S. enterica</i> serovar Typhimurium		
TR6579	Shuttle vector between <i>E. coli</i> and <i>S. enterica</i> serovar Typhimurium LT2	J. R. Roth
RMC26	<i>dxs</i> ::mevalonate operon (<i>araC P_{bad} erg8 erg12 erg19 Kan^r</i>)	C. A. Testa
RMC29	RMC26 with <i>idi</i> ::Cam ^r	This work
TT22236	<i>S. enterica</i> serovar Typhimurium (LT2) <i>cbiD24</i> ::MudJ containing pTP223	J. R. Roth
JMSB0351	RMC29 containing pJMSB0338	This work
JMSB0354	RMC29 containing pBAD Myc/HisA	This work
Plasmids		
pGEM-T Easy	Cloning vector for PCR products; Amp ^r	Promega
pBAD Myc/His A	<i>E. coli</i> expression vector; Amp ^r	Invitrogen
pJMSB0335	pGEM-T Easy with a 1.1-kb <i>Bgl</i> III- <i>Hind</i> III fragment corresponding to bp 26230 through 27279 in the <i>M. thermotrophicus</i> genome (ORF48)	This work
pJMSB0338	pBAD Myc/His A with a 1.1-kb <i>Bgl</i> III- <i>Hind</i> III fragment containing ORF48	This work
pTP223	<i>lam</i> , <i>bet</i> , and <i>exo</i> genes of phage lambda expressed from a <i>lac</i> promoter	A. R. Poteete and A. C. Fenton

^a Cam^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

Construction of strain RMC29 [*S. enterica* serovar Typhimurium LT2 *dxs::mevalonate operon (araC P_{bad} erg8 erg12 erg19 Kan) idi(del)::Cam^r(swap)*]. *Salmonella* cells expressing phage lambda recombination genes were used to facilitate linear transformation as described by Price-Carter et al. (25) and Yu et al. (46). A wild-type *Salmonella* strain carrying plasmid pTP223, strain TT22236, was used as a transformation recipient (25). Plasmid pTP223, supplied and constructed by Fenton and Poteete (8), expressed the phage lambda genes *exo*, *bet*, and *gam* from a *lac* promoter (25).

For construction of the *idi(del)::Cam^r(swap)* in a wild-type background, PCR primers were designed to amplify the CAM resistance (*Cam^r*) gene from pACYC184 while incorporating short flanking regions of homology. The 5' end of primer 1 included 40 bp of *Salmonella idi* sequence beginning 17 bases upstream of the start codon, followed by 20 bp of homologous sequence adjacent to the promoter of the *Cam^r* gene of pACYC184. The 5' end of primer 2 included 40 bp of complementary *idi* sequence beginning 7 bases downstream of the stop codon, followed by complementary sequence homologous to the region immediately following the *Cam^r* gene of pACYC184. The *Cam^r* gene of pACYC184 was amplified using these primers and *Taq* DNA polymerase. After purification, the resulting linear fragment was used to transform the recipient strain, TT22236, to CAM resistance. The resulting recombinants carried a *Cam^r* gene in place of the *idi* gene except for the upstream 23 bp and the downstream 33 bp of coding sequence. The insertion site was verified by PCR using primers flanking the expected insertion region followed by sequencing of the amplified DNA.

The construction of *S. enterica* serovar Typhimurium strain RMC26 is described elsewhere (40). RMC26 possesses a synthetic operon for the biosynthesis of IPP from mevalonic acid. The synthetic operon consists of the *Saccharomyces cerevisiae* genes *erg12* (mevalonate kinase), *erg8* (phosphomevalonate kinase), and *erg19* (diphosphomevalonate decarboxylase). These genes are controlled by an arabinose promoter, preceded by the *araC* gene and followed by a kanamycin resistance gene. The synthetic operon is inserted into a partially deleted chromosomal copy of *dxs*, the first gene in the MEP pathway. The resulting strain has an absolute requirement for exogenous supplementation with either MEV and AR or ME for the biosynthesis of essential isoprenoids. Strain RMC29 was constructed by crossing the *idi(del)::Cam^r(swap)* into strain RMC26 [*S. enterica* serovar Typhimurium LT2 *dxs::mevalonate operon (araC P_{bad} erg8 erg12 erg19 Kan)*] by standard transduction (40).

Construction of strain JMSB0354 (RMC29/pBADA Myc/His). Electrocompetent TR6579 *S. enterica* serovar Typhimurium cells, *metA22 metE551 trpD2 ilv-452 leu pro* (leaky) *hdsLT6 hdsSA29 hdsB-strA120 galE*, were electroporated with the expression vector pBAD Myc/His A from Invitrogen. Individual colonies from LB/AMP (LB plus AMP) agar plates were picked, and the plasmid DNA was isolated. The plasmid DNA was electroporated into freshly prepared electrocompetent RMC29 cells to create strain JMSB0354.

Construction of strain JMSB0351 (RMC29/pJMSB0338). Electrocompetent TR6579 cells were electroporated with pJMSB0338. Individual colonies from LB/AMP agar plates were picked, and the plasmid DNA was isolated. Strain JMSB0351 was created by electroporating the isolated plasmid DNA into electrocompetent strain RMC29.

Construction of *E. coli* strain JMSB0338 (BL21 DE3 pLysE/pJMSB0338). Electrocompetent BL21 DE3/pLysE cells were electroporated with pJMSB0338. Individual colonies from LB/AMP/CAM agar plates were picked. The plasmid DNA of these transformed colonies was isolated and sequenced for verification.

Expression of *M. thermautotrophicus* ORF48 and purification of the encoded protein. LB/AMP/CAM cultures (5 ml) were inoculated with single colonies of JMSB0338 and grown overnight at 37°C. These starter cultures were used to inoculate 500 ml of LB/AMP/CAM. The cultures were grown at 37°C to an A_{600} of approximately 0.6, L-arabinose was added to a final concentration of 0.2%, and incubation was continued for another 6 h. The cells were harvested by centrifugation.

All steps in the purification were performed at 4°C. Cell paste (~3 g) from strain JMSB0338 was suspended in 20 ml of lysis buffer consisting of 50 mM sodium phosphate (pH 8), 300 mM NaCl, and 10 mM imidazole. The cells were disrupted by sonication, and the resulting homogenate was centrifuged at 10,000 × *g* to remove cellular debris. Ni-NTA (5 ml, 50% slurry) was added to the cleared supernatant, and the suspension was swirled at 4°C at 100 rpm on a rotary shaker. The Ni-NTA and lysate were poured into a 100-ml fritted glass column, and the flowthrough was collected. The Ni-NTA resin was washed twice with 40 ml of a solution consisting of 20 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate (pH 8) and twice with 10 ml each of 78, 135, 192, and 250 mM imidazole (300 mM NaCl, 50 mM sodium phosphate [pH 8]). The eluates were collected separately and analyzed by SDS-PAGE. The purest fractions were

dialyzed against 50 mM HEPES (pH 6.8). The dialyzed protein was concentrated, yielding 195 µg of >90% purity.

Assays. Type II IPP isomerase activity was assayed by a modified version of the acid lability assay (30) described by Kaneda et al. (16). The assay cocktail consisted of 50 mM HEPES buffer (pH 6.8), 200 µM IPP (2 µCi/µmol), 20 µM FMN, 10 mM NADPH, 50 mM MgCl₂, and 50 µM dithiothreitol (DTT). The reaction was initiated by adding 10 µl of sample to 40 µl of the assay cocktail. The reaction mixture was incubated at 70°C for 10 min, quenched with 200 µl of methanol-HCl (4:1), and incubated for an additional 10 min at 37°C. Lignroine (1 ml), with a boiling point of 90 to 110°C, was added to the quenched reaction mixture and vortexed. A 500-µl portion of the organic layer was added to 10 ml of Cytosint scintillation fluid and counted by liquid scintillation spectrometry.

Purified type II IPP isomerase from *M. thermautotrophicus* was incubated in polybuffer with a pH range from 4.0 to 9.0, in 0.5 pH increments, for 30 min at room temperature and assayed as described above. The temperature dependence of the activity was determined by performing incubation at temperatures ranging from 22 to 95°C. FMN concentration was varied from 0 to 250 µM. NADPH concentration was varied from 0 to 20 mM. Divalent magnesium concentration was varied from 0 to 100 mM.

Identification of expressed *M. thermautotrophicus* ORF48. SDS-PAGE was performed on the Ni-NTA-purified protein from strain JMSB0338. A band corresponding to 50 kDa was cut from the denaturing 10% polyacrylamide gel. An in-gel tryptic digestion was performed on the excised band (13). The peptides generated from the proteolytic digestion were subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The mass-to-charge ratios of the isolated peptides were queried against the database using MS-Fit (5).

Product studies. A 500-µl sample containing 8 mM IPP or DMAPP, 65 nM isomerase, 10 mM NADPH, 20 µM FMN, 50 mM MgCl₂, and 1 mM DTT in 50 mM phosphate buffer (pH 7.0) was incubated for 15 h at 70°C. The sample was lyophilized, dissolved in D₂O, and analyzed by nuclear magnetic resonance (NMR) spectroscopy at 500 MHz.

Kinetic parameters. K_m and V_{max} for IPP were determined at various IPP concentrations between 10 and 800 µM [¹⁻¹⁴C]IPP (2 µCi/µmol). Assays of the initial velocities were performed in triplicate under optimal conditions as described above. Conversion of IPP to DMAPP was limited to 10% or less. K_m and V_{max} for IPP were obtained by fitting to the appropriate form of the Michaelis-Menten equation.

Sedimentation equilibrium. Sedimentation equilibrium experiments were conducted in a Beckman Optima XL-A analytical ultracentrifuge, with a Ti60 rotor at 20°C, using six-channel, 12-mm-thick charcoal-Epon centerpieces. The three sample channels in each cell contained three different loading concentrations of protein in 50 mM Tris, pH 7.5, with 150 mM NaCl and 25 µM DTT, while reference channels contained the dialysate. Loading concentrations varied between 1.12 and 4.47 µM. Cells were scanned radially in continuous mode, with data resulting from 10 absorbance readings taken at 0.001-cm intervals. Equilibrium was confirmed by no change in scans taken at 4 hourly intervals. Values of v -bar and the extinction coefficients for each protein were calculated from the amino acid sequence by the method of Laue et al. (21). Various models describing the concentration distribution were fit to final absorbance versus radius data using nonlinear least-squares techniques and the analysis program NONLIN (15, 45).

RESULTS

Archaeal genomes contain a gene for type II IPP isomerase.

Database searches were conducted for homology to the amino acid sequence of type II IPP isomerase from *Streptomyces* sp. strain CL190. Type II IPP isomerase ORFs were found in all of the archaeal genomes examined, including *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Methanothermobacter thermautotrophicus*, *Methanococcus jannaschii*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Sulfolobus solfataricus*, *Thermoplasma acidophilum*, *Pyrobaculum aerophilum*, *Pyrococcus furiosus*, *Sulfolobus tokodaii*, *Thermoplasma volcanium*, and *Halobacterium* sp. strain NRC-1. The archaeal IPP isomerases varied in length from 345 amino acids in *A. fulgidus* to 394 amino acids in *P. furiosus*.

A multiple-sequence alignment of the archaeal proteins revealed several conserved amino acids and regions of potential

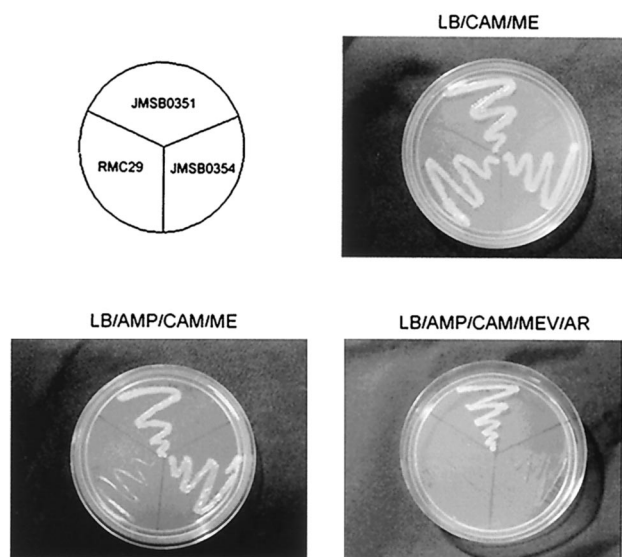


FIG. 2. Growth of *S. enterica* serovar Typhimurium strains JMSB0351, JMSB0354, and RMC29 on selective media.

significance. Type II IPP isomerase is classified in the NCBI database as a member of the 1304 cluster of orthologous groups (COG) (39) that includes L-lactate dehydrogenase (FMN dependent) and related alpha-hydroxy acid dehydrogenases. The inclusion of the type II IPP isomerases into COG 1304 is based on the strong specific alignment with the conserved domain for FMN-dependent dehydrogenases. There are several conserved glycine-rich regions that are likely part of the binding motifs for FMN and NADPH (42). Apart from the archaeal type II IPP isomerases, we identified additional homologous genes in the NCBI database using the *M. thermautotrophicus* sequence as a probe.

***M. thermautotrophicus* ORF48 complements IPP isomerase activity in vivo.** *S. enterica* serovar Typhimurium strain RMC29 differs from RMC26 by the deletion or insertion of a Cam^r cassette into the chromosomal copy of *idi*. Both RMC26 and RMC29 are viable when supplemented with ME through use of the MEP pathway genes downstream of *ispC* which biosynthesize IPP and DMAPP without the necessity of IPP isomerase activity. ME complements disruptions in *dxs* and *dxr* (9, 38). The alcohol is imported and phosphorylated before it enters the MEP pathway. Strain RMC26 is also viable in the presence of MEV, through utilization of both the synthetic operon, containing the yeast genes for the conversion of MEV to IPP, as well as the endogenous isomerase which converts IPP to DMAPP. However, RMC29 is not viable when supplemented with MEV due to the absence of endogenous isomerase activity; DMAPP is not produced in its absence. JMSB0351, JMSB0354, and RMC29 grew on LB/CAM/ME, demonstrating that all three strains do not require a functional IPP isomerase to synthesize DMAPP when utilizing the MEP pathway (Fig. 2) (27). Strains JMSB0354 and RMC29, which do not have a functional copy of *idi*, did not grow on LB/AMP/CAM/MEV/AR (Fig. 2). The disruption of chromosomal *idi* in JMSB0351 was complemented by a plasmid-encoded copy of ORF48 from *M. thermautotrophicus*. The *S. enterica* serovar

Typhimurium strain grew on LB/AMP/CAM/MEV/AR by utilizing the MVA pathway to synthesize IPP and the archaeal type II isomerase to convert IPP to DMAPP (Fig. 2).

***M. thermautotrophicus* type II IPP isomerase.** Type II IPP isomerase from *M. thermautotrophicus* was purified to >90% homogeneity in one step using Ni²⁺ agarose affinity chromatography. The expected molecular mass of the protein containing the C-terminal Myc/His tag from the pBADA Myc/His expression vector is 40,886 Da. A single band corresponding to ~48 kDa was cut from a SDS-polyacrylamide gel, and in-gel tryptic digestion was performed on the excised band (13). The peptides generated from the proteolytic digestion were subjected to MALDI-TOF mass spectrometry. The mass-to-charge ratios of the isolated peptides were queried against the database using MS-Fit (5). The resulting matched peptides corresponded to an excellent fit of the encoded protein from *M. thermautotrophicus* ORF48, with 39% of the amino acids covered, including major peaks in the mass spectrum at *m/z* 1148.614 for peptide KIDISLDFLGRE and *m/z* 1808.036 for peptide KSPVIITGHTGEWLNQRG.

Type II IPP isomerase activity was measured by the acid lability technique with the modifications offered by Kaneda et al. (16). The archaeal type II IPP isomerase from *M. thermautotrophicus* required the combined presence of FMN, NADPH, and Mg²⁺ for activity. The enzyme was maximally active at 70°C (Fig. 3).

The state of aggregation in solution for type II IPP isomerase from *M. thermautotrophicus* was examined using sedimentation equilibrium. In each case, all data were initially inspected for light redistribution at the base of the cell, and affected data were not included in the fitting process. The final fit used data from three different concentrations of protein. The fitting strategy involved fitting the various models of association to the concentration distributions using the program NONLIN. After final convergence, the baseline offset terms were included in the fit using a starting value measured by overspeeding the sample at the conclusion of the experiment. The best fit was obtained for a tetramer-octamer equilibrium, with a *K_D* of 17 μM. The data were well described by this model, as shown by the randomly distributed residuals (Fig. 4). At the concentration in the assays (29 nM), the protein exists in a homotetrameric state.

The products of the isomerization were determined by ¹H NMR spectroscopy. The enzyme, NADPH, and FMN were incubated with IPP or DMAPP. The sample was lyophilized, dissolved in D₂O, and analyzed. The conversion of IPP to DMAPP was confirmed by the appearance of peaks at 5.45 (H at C-2), 1.77 (E-methyl group), and 1.72 ppm (Z-methyl group) for IPP and a corresponding reduction in the intensity of peaks at 2.40 (H at C-2) and 1.78 ppm (methyl group) (35). Peaks for the protons at C-1 of both diphosphates and the C-4 olefinic protons in IPP were obscured.

The steady-state kinetic constants for *M. thermautotrophicus* type II IPP isomerase were as follows: *K_m* of 64 μM and *k_{cat}* of 1.6 s⁻¹. The catalytic efficiency (*k_{cat}*/*K_m*) of the enzyme was 2.5 × 10⁴ M⁻¹ s⁻¹. The kinetic parameters for the type II isomerases from *M. thermautotrophicus*, *Streptomyces* sp. strain CL190, and *Staphylococcus aureus* (16) and the type I isomerases from *E. coli* (10), *S. cerevisiae* (1), and *Homo sapiens* (11) are compared with type I isomerase from *M. thermautotrophici-*

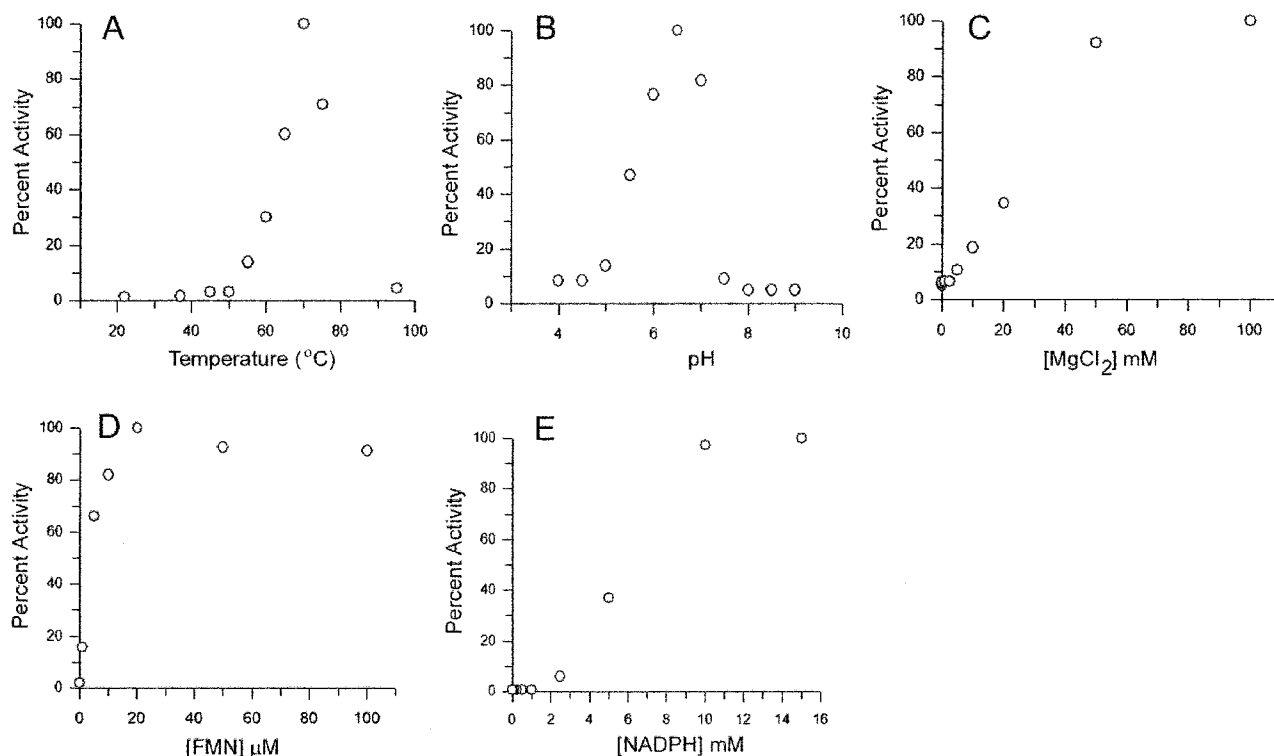


FIG. 3. Dependence of type II IPP isomerase activity from *M. thermautotrophicus* on temperature (A), pH (B), $[MgCl_2]$ (C), $[FMN]$ (D), and $[NADPH]$ (E).

cus in Table 2. Except for the *Streptomyces* enzyme, the catalytic efficiencies of the type I and type II IPP isomerases from widely divergent organisms are similar.

DISCUSSION

Isoprenoid molecules are required to support life, and all organisms must synthesize isoprenoid molecules *de novo* to survive. The *Archaea* have a unique, signature requirement for isoprenoids during the biosynthesis of their cellular membranes (41). The membrane lipids of *M. thermautotrophicus* consist of C_{40} and C_{80} glyceryl ethers where the alkyl moieties are methyl branched isoprenoid chains (23). The basic membrane lipid is a C_{40} diether consisting of two C_{20} phytanyl chains ether linked to C-2 and C-3 of glycerol. The tetraethers are macrocyclic molecules formed by cross-linking the ends of the phytanyl chains of two diether molecules (12, 23). The proportion of tetraethers in membrane lipids increases with increases in temperature (14) and contributes to the robust nature of archaeal membranes designed to function in extreme environments (17).

Labeling studies indicate that archaeal membrane ethers are synthesized from acetyl coenzyme A (acetyl-CoA) by the MVA pathway (24). Thus far, putative genes for acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and MVA kinase have been identified in the *M. thermautotrophicus* chromosome (20). Interestingly, homologs for PMVA kinase and DPMVA decarboxylase have not yet been found in the DNA database for the archaeon.

IPP isomerase activity is essential to convert IPP to DMAPP for isoprenoids synthesized by the MVA pathway. The type I enzyme fulfills this function in eukaryota and some members of the domain *Bacteria*. Type I IPP isomerase is monomeric and requires Mg^{2+} or Mn^{2+} for activity. Affinity labeling (37) and site-directed mutagenesis (36) experiments reveal that the enzyme has essential active-site glutamic acid and cysteine residues. These amino acids are thought to be involved in the addition and abstraction of protons during the isomerization of IPP to DMAPP by a proton addition-elimination mechanism (36). Recent crystal structures of type I IPP isomerase from *E. coli* (43, 44) indicate that the active enzyme has two metals in the active site. One is presumably magnesium, which stabilizes interactions between the diphosphate residue and the protein. The other metal is coordinated by three histidines and two glutamates, one of which was previously implicated in catalysis by the affinity labeling and site-directed mutagenesis.

Homologs for type II IPP isomerase have been found in all archaeal chromosomes sequenced so far and in some eubacteria (mostly proteobacteria, cyanobacteria, and gram-positive eubacteria). In contrast to the type I enzyme, type II IPP isomerase exists in a homotetrameric state in solution, requiring FMN, NADPH, and Mg^{2+} as cofactors. Although the chemical mechanism for the isomerization catalyzed by the type II enzyme is not known, it is undoubtedly different than the protonation or deprotonation sequence employed by type I IPP isomerase.

IPP isomerase activity was first detected in the cellular lysate of *M. thermautotrophicus* in 1993 (47). The genome of the

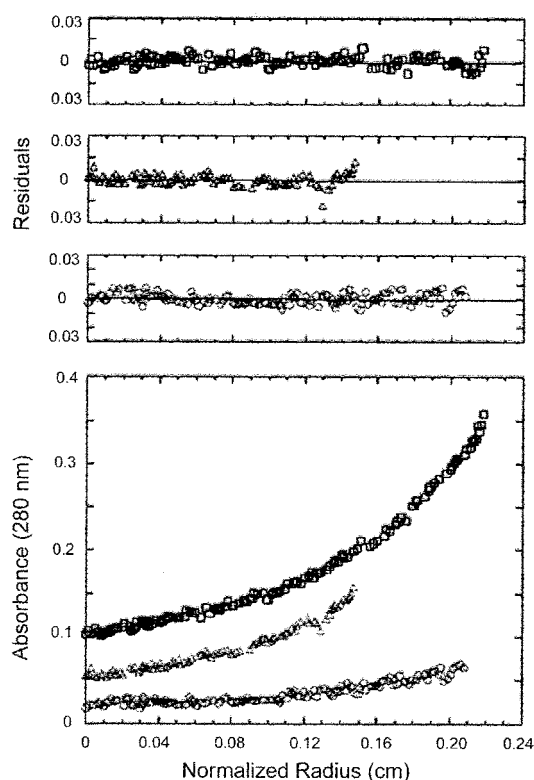


FIG. 4. Sedimentation equilibrium data for type II IPP isomerase from *M. thermautotrophicus*. The lower panel shows experimental data points for three different loading concentrations ($\square$, 4.47 μM ; $\triangle$, 2.24 μM ; $\circ$, 1.12 μM) of the protein. The upper panels show the residuals for fits of experimental data to the tetrameric model. The small and random deviations indicate a good fit corresponding to a K_D of 17 μM .

archaeon was sequenced in 1997 before the discovery of the type II enzyme, and it was somewhat surprising that no homologue of the known type I IPP isomerase was found. After bioinformatic approaches failed to find an *idi* gene in *Archaea*, it was suggested that archaeal IPP isomerase might be a novel enzyme (33). Following the discovery of type II IPP isomerase in *Streptomyces* sp. strain CL190 (16), several ORFs that encoded proteins homologous proteins were found in *Archaea*, including ORF48 in *M. thermautotrophicus*.

TABLE 2. Kinetic parameters for isomerases across the three kingdoms

Organism	IPP K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}/\text{s}^{-1}$)	Type
<i>M. thermautotrophicus</i> ^a	64	1.6	5.0×10^4	II
<i>S. aureus</i> ^b	19	1.3	6.8×10^4	II
<i>Streptomyces</i> sp. strain CL190 ^b	450	0.70	1.6×10^3	II
<i>H. sapiens</i> ^c	33	1.8	5.5×10^4	I
<i>S. cerevisiae</i> ^d	43	8.0	1.9×10^5	I
<i>E. coli</i> ^e	7.9	0.33	4.2×10^4	I

^a Data from this work.

^b Data from reference 16.

^c Data from reference 11.

^d Data from reference 1.

^e Data from reference 10.

We have now established that ORF48 encodes a type II IPP isomerase. The archaeal gene complements an IPP isomerase deletion in a strain of *S. enterica* serovar Typhimurium specifically engineered to use either methyl erythritol or mevalonate as the sole precursor for isoprenoid biosynthesis. When complemented with ME, isomerase activity is not required for growth. This observation is consistent with the recently established ability of the *ispH* gene product to synthesize both IPP and DMAPP in the last step of the MEP pathway (27). In contrast, a functional IPP isomerase is essential when the strain is grown on media supplemented with only mevalonate. Interestingly, the *M. thermautotrophicus* enzyme was sufficiently active at 37°C to complement the disruption of the *idi* gene in *S. enterica* serovar Typhimurium. *M. thermautotrophicus* is a moderate thermophile, and the optimal temperature for the enzyme is ~70°C (32). The activity of the enzyme is substantially lower at 37°C.

In summary, ORF48 from *M. thermautotrophicus* encodes type II IPP isomerase. The recombinant protein was characterized by MALDI mass spectrometry of peptide fragments generated by in-gel tryptic digestion. ORF48 complemented the disruption of *idi* in a *S. enterica* serovar Typhimurium mutant strain engineered to synthesize IPP from MEV. IPP isomerase activity for the archaeal enzyme was also established biochemically. IPP isomerases have now been established in organisms across the three kingdoms of life.

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REFERENCES

- Anderson, M. S., M. Muchlbacher, I. P. Street, J. Proffitt, and C. D. Poulter. 1989. Isopentenyl diphosphate:dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**:19169–19175.
- Bobik, T. A., M. Ailion, and J. R. Roth. 1992. A single regulatory gene integrates control of vitamin B₁₂ synthesis and propanediol degradation. *J. Bacteriol.* **174**:2253–2266.
- Buckingham, J. 1998. Dictionary of natural products on CD-ROM, version 6.1. Chapman & Hall, Ltd., London, England.
- Chaykin, S., J. Law, A. H. Phillips, T. T. Tchen, and K. Bloch. 1958. Phosphorylated intermediates in the synthesis of squalene. *Biochemistry* **44**:998–1003.
- Clauser, K. R., P. Baker, and A. L. Burlingame. 1999. Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem.* **71**:2871–2882.
- Cornforth, J. W., and G. Popjak. 1959. Mechanism of biosynthesis of squalene from sesquiterpenoids. *Tetrahedron Lett.* **19**:29–35.
- Duvold, T., P. Cali, J. M. Bravo, and M. Rohmer. 1997. Incorporation of 2-C-methyl-D-erythritol, a putative isoprenoid precursor in the mevalonate-independent pathway, into ubiquinone and menaquinone of *Escherichia coli*. *Tetrahedron Lett.* **38**:6181–6184.
- Fenton, A. C., and A. R. Poteete. 1984. Genetic analysis of the *erf* region of the bacteriophage P22 chromosome. *Virology* **134**:148–160.
- Hahn, F. M., L. M. Eubanks, C. A. Testa, B. S. Blagg, J. A. Baker, and C. D. Poulter. 2001. 1-Deoxy-D-xylulose 5-phosphate synthase, the gene product of open reading frame (ORF) 2816 and ORF 2895 in *Rhodobacter capsulatus*. *J. Bacteriol.* **183**:1–11.
- Hahn, F. M., A. P. Hurlburt, and C. D. Poulter. 1999. *Escherichia coli* open reading frame 696 is *idi*, a nonessential gene encoding isopentenyl diphosphate isomerase. *J. Bacteriol.* **181**:4499–4504.
- Hahn, F. M., J. W. Xuan, A. F. Chambers, and C. D. Poulter. 1996. Human isopentenyl diphosphate:dimethylallyl diphosphate isomerase: overproduction, purification, and characterization. *Arch. Biochem. Biophys.* **332**:30–34.
- Heathcock, C. H., B. L. Finkelstein, T. Aoki, and C. D. Poulter. 1985. Stereostructure of the archaeobacterial C40 diol. *Science* **229**:862–864.

13. Hellman, U., C. Wernstedt, J. Genez, and C. H. Heldin. 1995. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* **224**:451–455.
14. Itoh, Y. H., A. Sugai, I. Uda, and T. Itoh. 2001. The evolution of lipids. *Adv. Space Res.* **28**:719–724.
15. Johnson, M. L., J. J. Correia, D. A. Yphantis, and H. R. Halvorson. 1981. Analysis of data from the analytical ultracentrifuge by nonlinear least-squares techniques. *Biophys. J.* **36**:575–588.
16. Kaneda, K., T. Kuzuyama, M. Takagi, and H. Seto. 2002. An unusual isopentenyl diphosphate isomerase found in the mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190. *Proc. Natl. Acad. Sci. USA* **98**:932–937.
17. Koga, Y., M. Nishihara, H. Morii, and M. Akagawa-Matsushita. 1993. Ether polar lipids of methanogenic bacteria: structures, comparative aspects, and biosyntheses. *Microbiol. Rev.* **57**:164–182.
18. Kuzuyama, T., and H. Seto. 2003. Diversity of the biosynthesis of the isoprene units. *Nat. Prod. Rep.* **20**:171–183.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
20. Lange, B. M., T. Rujan, W. Martin, and R. Croteau. 2000. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. USA* **97**:13172–13177.
21. Laue, T. M., B. D. Shah, T. M. Ridgeway, and S. L. Pelletier. 1991. Computer-aided interpretation of analytical sedimentation data for proteins, p. 90–125. *In* S. E. Harding, A. J. Rowe, and J. C. Horton (ed.), *Analytical ultracentrifugation in biochemistry and polymer science*. The Royal Society of Chemistry, Cambridge, United Kingdom.
22. Leatherbarrow, R. J. 1992. Graft version 5.0.3. Erithicus Software Ltd., Staines, England.
23. Nishihara, M., H. Morii, and Y. Koga. 1989. Heptads of polar ether lipids of an archaeobacterium, *Methanobacterium thermoautotrophicum*: structure and biosynthetic relationship. *Biochemistry* **28**:95–102.
24. Poulter, C. D., T. Aoki, and L. Daniels. 1988. Biosynthesis of isoprenoid membranes in the methanogenic archaeobacterium *Methanospirillum hungatei*. *J. Am. Chem. Soc.* **110**:2620–2624.
25. Price-Carter, M., J. Tingey, T. A. Bobik, and J. R. Roth. 2001. The alternative electron acceptor tetrathionate supports B₁₂-dependent anaerobic growth of *Salmonella enterica* serovar Typhimurium on ethanolamine or 1,2-propanediol. *J. Bacteriol.* **183**:2463–2475.
26. Rodriguez-Concepcion, M., N. Campos, L. L. Maria, C. Maldonado, J. F. Hoeffler, C. Grosdemange-Billiard, M. Rohmer, and A. Boronat. 2000. Genetic evidence of branching in the isoprenoid pathway for the production of isopentenyl diphosphate and dimethylallyl diphosphate in *Escherichia coli*. *FEBS Lett.* **473**:328–332.
27. Rohdich, F., S. Hecht, K. Gartner, P. Adam, C. Krieger, S. Amslinger, D. Arigoni, A. Bacher, and W. Eisenreich. 2002. Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein. *Proc. Natl. Acad. Sci. USA* **99**:1158–1163.
28. Rohmer, M. 1999. Isoprenoids including carotenoids and steroids, p. 45–67. *In* *Comprehensive natural products chemistry*, vol. 2. Isoprenoids including carotenoids and steroids. Elsevier, Amsterdam, The Netherlands.
29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. Satterwhite, D. M. 1985. Steroids and isoprenoids. *Methods Enzymol.* **110**: 92–99.
31. Schmieger, H. 1971. A method for detection of phage mutants with altered transducing ability. *Mol. Gen. Genet.* **110**:378–381.
32. Schonheit, P., J. Moll, and R. K. Thauer. 1980. Growth parameters of *Methanobacterium thermoautotrophicum*. *Arch. Microbiol.* **127**:59–65.
33. Smit, A., and A. Mushegian. 2000. Biosynthesis of isoprenoids via mevalonate in Archaea: the lost pathway. *Genome Res.* **10**:1468–1484.
34. Smith, D. R., L. A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Spadafora, R. Vicaire, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, H. Safer, D. Patwell, S. Prabhakar, S. McDougall, G. Shimer, A. Goyal, S. Pietrowski, G. M. Church, C. J. Daniels, J.-I. Mao, P. Rice, J. Nöling, and J. N. Reeve. 1997. Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. *J. Bacteriol.* **179**:7135–7155.
35. Street, I. P., D. J. Christensen, and C. D. Poulter. 1990. Hydrogen exchange during the enzyme-catalyzed isomerization of isopentenyl diphosphate and dimethylallyl diphosphate. *J. Am. Chem. Soc.* **112**:8577–8578.
36. Street, I. P., H. R. Coffman, C. D. Poulter, and J. A. Baker. 1994. Identification of Cys139 and Glu207 as catalytically important groups in the active site of isopentenyl diphosphate:dimethylallyl diphosphate isomerase. *Biochemistry* **33**:4212–4217.
37. Street, I. P., and C. D. Poulter. 1990. Isopentenyl diphosphate:dimethylallyl diphosphate isomerase: construction of a high-level heterologous expression system for the gene from *Saccharomyces cerevisiae* and identification of an active-site nucleophile. *Biochemistry* **29**:7531–7538.
38. Takahashi, S., H. Watanabe, and H. Seto. 1998. Direct formation of the 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate by 1-deoxy-D-xylulose 5-phosphate reductoisomerase, a new enzyme in the non-mevalonate pathway to isopentenyl diphosphate. *Tetrahedron Lett.* **39**: 4509–4512.
39. Tatusov, R. L., D. A. Natale, I. V. Garkavtsev, T. A. Tatusova, U. T. Shankavaram, B. S. Rao, B. Kiryutin, M. Y. Galperin, N. D. Fedorova, and E. V. Koonin. 2001. The COG database: new developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Res.* **29**:22–28.
40. Testa, C. A., R. M. Cornish, and C. D. Poulter. 2004. The sorbitol phosphotransferase system is responsible for the transport of exogenous 2-C-methyl-D-erythritol into *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **186**: 473–480.
41. van de Vossenberg, J. L., A. J. Driessen, and W. N. Konings. 1998. The essence of being extremophilic: the role of the unique archaeal membrane lipids. *Extremophiles* **2**:163–170.
42. Wang, M., D. L. Roberts, R. Paschke, T. M. Shea, B. S. Masters, and J. J. Kim. 1997. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc. Natl. Acad. Sci. USA* **94**:8411–8416.
43. Wouters, J., Y. Oudjama, S. J. Barkley, C. Tricot, V. Stalon, L. Droogmans, and C. D. Poulter. 2003. Catalytic mechanism of *Escherichia coli* isopentenyl diphosphate isomerase involves Cys-67, Glu-116, and Tyr-104 as suggested by crystal structures of complexes with transition state analogues and irreversible inhibitors. *J. Biol. Chem.* **278**:11903–11908.
44. Wouters, J., Y. Oudjama, S. Ghosh, V. Stalon, L. Droogmans, and E. Oldfield. 2003. Structure and mechanism of action of isopentenylpyrophosphate-dimethylallylpyrophosphate isomerase. *J. Am. Chem. Soc.* **125**:3198–3199.
45. Yphantis, D. A. 1964. Equilibrium ultracentrifugation of dilute solutions. *Biochemistry* **3**:297–317.
46. Yu, D., H. M. Ellis, E.-C. Lee, N. A. Jenkins, N. G. Copeland, and D. Court. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97**:5978–5983.
47. Zhang, D., and C. D. Poulter. 1993. Biosynthesis of archaeobacterial lipids in *Halobacterium halobium* and *Methanobacterium thermoautotrophicum*. *J. Org. Chem.* **58**:3919–3922.