Identification of a novel class of *ω,E,E*-farnesyl diphosphate synthase from *Mycobacterium tuberculosis*

Rakesh K. Dhiman,* Mark C. Schulbach,* Sebabrata Mahapatra,* Alain R. Baulard,† Varalakshmi Vissa,* Patrick J. Brennan,* and Dean C. Crick*,[1]

Department of Microbiology,* Colorado State University, Fort Collins, CO 80523-1677; and Institut National de la Santé et de la Recherche Médicale U447,† Institut Pasteur de Lille, 59019 Lille cedex, France

Abstract **We have identified an *ω,E,E*-farnesyl diphosphate (*ω,E,E*-FPP) synthase, encoded by the open reading frame *Rv3398c*, from *Mycobacterium tuberculosis* that is unique among reported FPP synthases in that it does not contain the type I (eukaryotic) or the type II (eubacterial) *ω,E,E*-FPP synthase signature motif. Instead, it has a structural motif similar to that of the type I geranylgeranyl diphosphate synthase found in *Archaea*. Thus, the enzyme represents a novel class of *ω,E,E*-FPP synthase. *Rv3398c* was cloned from the *M. tuberculosis* H37Rv genome and expressed in *Mycobacterium smegmatis* using a new mycobacterial expression vector (pVV2) that encodes an in-frame N-terminal affinity tag fusion with the protein of interest. The fusion protein was well expressed and could be purified to near homogeneity, allowing facile kinetic analysis of recombinant Rv3398c. Of the potential allylic substrates tested, including dimethylallyl diphosphate, only geranyl diphosphate served as an acceptor for isopentenyl diphosphate. The enzyme has an absolute requirement for divalent cation and has a K_m of 43 μM for isopentenyl diphosphate and 9.8 μM for geranyl diphosphate and is reported to be essential for the viability of *M. tuberculosis*.**—Dhiman, R. K., M. C. Schulbach, S. Mahapatra, A. R. Baulard, V. Vissa, P. J. Brennan, and D. C. Crick. **Identification of a novel class of *ω,E,E*-farnesyl diphosphate synthase from *Mycobacterium tuberculosis*. *J. Lipid Res.* 2004.** 45: **1140–1147.**

Supplementary key words dimethylallyl diphosphate • mycobacterial expression vector • short-chain prenyl diphosphate synthase

ω,E,E-farnesyl diphosphate (*ω,E,E*-FPP) is typically synthesized by short-chain isoprenyl diphosphate synthases that catalyze an electrophilic alkylation reaction of isopentenyl diphosphate (IPP) to an allylic diphosphate such as dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) (**Fig. 1**). To date, amino acid sequence alignments have been generated for more than 50 *E*-isoprenyl diphosphate synthases from diverse sources (1–3). These studies have identified five regions of amino acid conservation, including two aspartate-rich motifs that are referred to as the first and second aspartate-rich motifs. The motifs are necessary for binding the diphosphate moieties of the substrates (4), and site-directed mutagenesis experiments have defined key amino acids around the first aspartate-rich motif (FARM) that determine the chain length of the product (5–8). These amino acids constitute the chain length-determining (CLD) region.

In a recent review, the CLD region and the FARM signatures were used to segregate the *ω,E,E*-FPP synthases into two classes designated type I (eukaryotic) and type II (eubacterial) (1). Both of these classes of enzyme presumably evolved from a common precursor similar to the archaeal type I geranylgeranyl diphosphate (GGPP) synthase, which contains the FARM signature DDXXD and a single aromatic amino acid in the fifth position N-terminal to the FARM. Type I *ω,E,E*-FPP synthases (eukaryotic) are similar to the type I GGPP synthases in that they also contain the DDXXD motif. However, they have two aromatic amino acids at the fourth and fifth positions N-terminal to the FARM. Type II *ω,E,E*-FPP synthases (eubacterial) contain the FARM signature DDXXXXD and a single aromatic amino acid in the fifth position N-terminal to the FARM. This classification system is now well established and is being used to identify putative prenyl diphosphate synthases as FPP or GGPP synthases for phylogenetic studies (9, 10) and genome annotation.

In the course of work on the synthesis of *ω,E,Z*-farnesyl diphosphate (*ω,E,Z*-FPP), an intermediate in the synthesis of decaprenyl phosphate of *Mycobacterium tuberculosis* (11), we also observed the synthesis of *ω,E,E*-FPP. This observation, combined with a report of sterol synthesis in mycobacteria (12), prompted us to try to identify the enzyme

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Abbreviations: BLAST, Basic Local Alignment Search Tool; CLD, chain length-determining; DMAPP, dimethylallyl diphosphate; FARM, first aspartate-rich motif; FOH, farnesol; FPP, farnesyl diphosphate; GGOH, geranylgeraniol; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP, isopentenyl diphosphate; LB, Luria-Bertani; NCBI, National Center for Biotechnology Information.

[1] To whom correspondence should be addressed.
e-mail: dean.crick@colostate.edu

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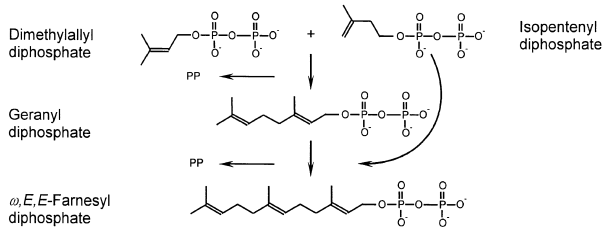

Figure 1. Reactions catalyzed by *ω,E,E*-farnesyl diphosphate (*ω,E,E*-FPP) synthases.

responsible for the synthesis of *ω,E,E*-FPP in *M. tuberculosis*. However, Basic Local Alignment Search Tool (BLAST) searches of the *M. tuberculosis* genome (13) did not reveal any genes encoding enzymes with amino acid sequences that conform to the known features of eubacterial type II *ω,E,E*-FPP synthases. These data show that the *M. tuberculosis* open reading frame *Rv3398c* encodes a unique *ω,E,E*-FPP synthase. To date, it is the only reported *ω,E,E*-FPP synthase that does not fit the established classification system in that it has the type I aspartate motif in the FARM and the CLD region characteristic of archaeal type I GGPP synthases.

MATERIALS AND METHODS

Materials

[^{14}C]IPP (55 mCi/mmol) was purchased from Amersham Life Science, Inc., and potato acid phosphatase (grade 2) was purchased from Roche Applied Science. Kanamycin, DMAPP, GGPP, farnesol (FOH; mixed stereoisomers), *ω,E,E*-FOH, *ω,E*-geraniol, *ω,E,E,E*-geranylgeraniol (GGOH), monoclonal anti-polyhistidine from mouse ascites fluid, anti-mouse IgG conjugated to alkaline phosphatase, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets were purchased from Sigma. *ω,E,E*-FPP and GPP were synthesized as previously described (14). *ω,E,E,Z*-GGOH was a gift from Dr. C. J. Waechter (University of Kentucky). Authentic prenols of various chain lengths were purchased from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Kieselgel 60 F254 TLC plates were from EM Science, and Baker Si-Reverse Phase C_{18} TLC plates were from J. T. Baker. *Eco*RI, *Msc*I, Luria-Bertani (LB) agar and LB broth, deoxynucleotide triphosphates, T4 DNA ligase, and *Escherichia coli* DH5α were purchased from Invitrogen. Vent polymerase, *Nde*I, *Bam*HI, and *Hin*dIII were from New England Biolabs, Inc. QIAprep Spin Miniprep Kits were purchased from Qiagen, Inc., and *E. coli* competent cells (XL-1 Blue) were purchased from Stratagene. All other supplies and reagents were of analytical grade and from standard sources unless indicated otherwise.

Cloning and expression of native *Rv3398c*

The following primers were designed to amplify open reading frame *Rv3398c* from *M. tuberculosis* H37Rv genomic DNA: 5′-GCGGTACAGACGAAAAGTACGGACTGC-3′ and 5′-ACGCCTGTTCGAATTCGGTCATGC-3′. An *Eco*RI restriction endonuclease site (underlined) was engineered into the C-terminal primer. PCR was performed using a Perkin-Elmer GeneAmp 2400 PCR System and Vent DNA Polymerase. The PCR products were digested with *Eco*RI and ligated into the mycobacterial expression vector pMV261, which had been previously digested with *Msc*I and *Eco*RI. The ligation mixture was used to transform chemically competent *E. coli* XL-1 Blue cells. Clones were selected on LB agar plates containing kanamycin at 20 μg/ml. Plasmids were purified and confirmed by restriction and sequence analysis. The construct, named pMV-Rv3398c, and pMV261 (empty vector) were electroporated into *M. smegmatis* $mc^2$155 (American Type Culture Collection). Electroporation-competent *M. smegmatis* cells were prepared by growing a culture to mid-log phase in LB broth and washing seven times with ice-cold, sterile 10% glycerol; competent cells were stored at −70°C. Electroporation was performed at 2.5 kV, 800 Ω, and 25 μF. Cells were allowed to recover in LB broth for 3 h and were plated on LB agar containing kanamycin (20 μg/ml). A single colony was used to start a liquid culture in LB broth containing kanamycin (20 μg/ml).

Subcellular fractionation

Wild-type and transformed *M. smegmatis* strains (containing pMV261 or pMV261-Rv3398c) were grown to mid-log phase in LB broth (containing 20 μg/ml kanamycin for transformed strains) and harvested by centrifugation. Cells were disrupted by probe sonication on ice (Sanyo Soniprep 150, 10 cycles of 60 s on and 90 s off) in a buffer containing 50 mM MOPS (pH 7.9), 0.25 M sucrose, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. The resulting suspension was centrifuged at 15,000 *g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 200,000 *g* for 1 h in a Beckman 70.1Ti rotor. The supernatant (cytosol) was stored in 1 ml aliquots and stored at −70°C. The pellets (membranes) were resuspended in the homogenization buffer and stored as aliquots at −70°C.

Engineering a vector for the expression of N-terminal histidine-tagged protein in mycobacteria

To enable the expression of fusion proteins with six histidine residues at the N-terminus, a new vector was engineered from pMV261 (15) that uses the same selectable marker (kanamycin), promoter, and ribosome binding sequences for the expression of cloned genes. A short oligonucleotide cassette was obtained by annealing the complementary oligonucleotides, 5′-TAATGCGCGGCTCGCACCACCACCACCACCATATGGCCAAGACAATTGCG-3′ and 5′-GATCCGCAATTGTCTTGGCCATATGGTGGTGGTGGTGGTGCGAGCCGCGCAT-3′, in a mixture containing 40 μM of each of the oligonucleotides, 20 mM $MgCl_2$, 200 mM NaCl, and 100 mM Tris-HCl (pH 8). The 50 μl volume was incubated at 100°C for 10 min, cooled to 65°C, and finally allowed to cool to room temperature. The annealed cassette, with *Nde*I- and *Bam*HI-compatible 5′ overhangs, was ligated between the *Nde*I and *Bam*HI restriction sites of pJJV7, a derivative of pMV261 previously engineered to contain a *Nde*I restriction site. After transformation of the ligation mixture into chemically competent *E. coli* DH5α cells, several kanamycin-resistant clones were picked and the plasmid DNA was isolated and subjected to restriction analysis. The region including the promoter, multiple cloning sites, and N-terminal histidine tag of the plasmid (pVV2) was verified by sequencing. Ligation of the 5′ end of the gene of interest at the *Nde*I site creates an in-frame N-terminal fusion with the sequence MGRSHHHHHH encoded by the vector.

Expression and purification of affinity-tagged Rv3398c

The following primers were designed to amplify open reading frame *Rv3398c* from *M. tuberculosis* H37Rv genomic DNA: 5′-ATTCACCATATGCGCGGTACCGACGAAAA-3′ and 5′-ACGGAAGCTTTCAGTCACGCCTGACGATC-3′. *Nde*I and *Hin*dIII restriction endonuclease sites (underlined) were engineered into the primers. PCR was performed using a Perkin-Elmer Gene Amp

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2400 PCR machine and Vent DNA polymerase. The PCR-amplified product was digested with *Nde*I and *Hind*III and ligated to pET-28a(+). This construct was used to transform *E. coli* (DH5α) cells. Bacteria containing plasmid were selected on LB agar containing kanamycin (50 μg/ml). Plasmids were purified and subjected to restriction and sequence analysis. Plasmids were then digested with *Nde*I and *Hind*III, and the fragment containing *Rv3398c* was gel purified and ligated to pVV2. This construct (pVV2-Rv3398c) was propagated in *E. coli* DH5α. Electrocompetent *M. smegmatis mc*^{2}155 cells were prepared and transformed as described above. Cells containing plasmid were allowed to recover in LB broth for 3 h and plated on LB agar plates containing 50 μg/ml kanamycin. A single colony was chosen to start a liquid culture in LB broth containing 50 μg/ml kanamycin.

Cells were harvested from a 1 liter culture by centrifugation, washed, suspended in 50 mM MOPS (pH 8), 10 mM $MgCl_2$, 500 mM NaCl, 15% glycerol, and 5 mM 2-mercaptoethanol, and disrupted by probe sonication as described earlier. The sonicate was then centrifuged at 27,000 *g*. The cleared supernatant was loaded on a Talon metal affinity resin column (Clontech) or a HiTrap chelating HP column (Amersham Biosciences) that was preequilibrated with 50 mM MOPS (pH 8), 1.0 mM $MgCl_2$, 500 mM NaCl, 15% glycerol, and 5 mM 2-mercaptoethanol (buffer A) for purification on a Bio-Rad Duoflow liquid chromatography system. The columns were washed with 10 volumes of buffer A containing 10 mM imidazole. Bound protein was eluted using either a step gradient of buffer A containing 50, 100, 200, or 400 mM imidazole or a linear gradient of imidazole from 0 to 400 mM. Fractions were collected, and the protein was analyzed by SDS-PAGE and Western blotting. Fractions containing purified, recombinant Rv3398c were pooled and desalted. The pooled material was judged to be at least 90% pure by SDS-PAGE analysis using Coomassie Brilliant Blue R250 to visualize the protein. Aliquots were frozen and stored at −70°C for later use.

In vitro FPP synthase assays

FPP synthase activity was assayed in mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2% Triton X-100, and the indicated concentrations of allylic diphosphate, [^{14}C]IPP, and protein (cytosolic, membrane, or recombinant) in a final volume of 50 μl. After incubating at 37°C for an appropriate length of time, the reaction was stopped by the addition of 1 ml of water saturated with NaCl. Radiolabeled products were extracted with butanol saturated with water, and an aliquot was taken for liquid scintillation spectrometry. Reactions were assayed under conditions in which product formation was linear for both time and protein concentration.

Enzymatic dephosphorylation of reaction products

Butanol was removed under a stream of nitrogen, and the radiolabeled products were dissolved in 5 ml of buffer containing 100 mM sodium acetate (pH 4.8), 0.1% Triton X-100, and 60% methanol (16). After a brief bath sonication, 20 U of potato acid phosphatase was added and the mixture was incubated at 25°C overnight. Dephosphorylated products were extracted three times with 1 ml of *n*-hexane. The pooled extracts were washed with 1 ml of water, and the solvent was evaporated under nitrogen. The samples were dissolved in 200 μl of chloroform-methanol (2:1, v/v), and an aliquot was taken for liquid scintillation spectrometry. A second aliquot was used for TLC analysis.

Analysis of radiolabeled product chain length and stereochemistry

Analysis of the chain length of the dephosphorylated products was accomplished by reverse-phase C_{18} TLC as previously described (11). The plates were developed in methanol-acetone (8:2, v/v), and the radioactive products were located with a Bioscan System 200 Imaging Scanner (Bioscan, Inc.) and by autoradiography. Standard polyprenols were located with an anisaldehyde spray reagent (17).

The stereochemistry of radiolabeled products was determined as previously described (18, 19). Briefly, the radioactive products derived from enzymatically labeled, dephosphorylated products that were identified as FOH or GGOH were scraped from the reverse-phase TLC plates and extracted from the gel with two 5 ml volumes of chloroform-methanol (2:1, v/v). The extracts were pooled and dried under nitrogen. Radiolabeled FOH was dissolved in chloroform-methanol (2:1, v/v) containing authentic, nonradioactive *ω,E,E*-FOH or mixed stereoisomers (*ω,E,E* and *ω,E,Z*) of FOH. Radiolabeled GGOH was dissolved in chloroform-methanol (2:1, v/v) containing authentic, nonradioactive *ω,E,E,E*-GGOH or *ω,E,E,Z*-GGOH. The stereochemistry of the radiolabeled product was determined by silica gel 60 TLC developed with toluene-ethyl acetate (7:3, v/v). Radioactive spots were located with the Bioscan System 200 Imaging Scanner and by autoradiography. Standard polyprenols were located with an anisaldehyde spray reagent (17).

Amino acid sequence analysis

Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) World Wide Web site. BLAST searches were also performed at the NCBI site. Amino acid sequence alignments were performed on the Multalin interface (World Wide Web) using hierarchical clustering (20). Clustal W analysis was performed on the European Molecular Biology Network (EMBnet) World Wide Web site, and the data were graphically displayed using TreeView version 1.6.1.

Other analytical procedures

The protein concentrations of the fractions were estimated using a bicinchoninic acid protein assay kit (Pierce). SDS-PAGE analysis was done using 10% polyacrylamide gels with 5% stacking gels, and in some cases proteins were electroblotted to Protran nitrocellulose membranes (Schleicher and Schuell). Polyhistidine-tagged recombinant proteins were visualized using monoclonal anti-polyhistidine from mouse ascites fluid as a primary antibody, anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets according to the manufacturer's instructions. DNA sequencing and oligonucleotide synthesis were done by Macromolecular Resources at Colorado State University. Kinetic constants were calculated by nonlinear regression analysis using GraFit 5 (Erithacus Software).

RESULTS

Expression of Rv3398c increases the incorporation of [^{14}C]IPP into butanol-extractable material

Rv3398c was cloned into pMV261 and expressed in *M. smegmatis*. Cytosolic and membrane fractions from the wild-type and transformed bacteria were assayed for [^{14}C]IPP incorporation into butanol-extractable material in the presence of various allylic primers. The specific activity of the Rv3398c recombinant cytosol preparation primed with DMAPP or GPP was 2-fold higher than that of the corresponding wild-type cytosol (data not shown).

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Chain length analysis of the products synthesized by *M. smegmatis* cytosol harboring pMV-Rv3398c

The products of the cytosolic assays were enzymatically dephosphorylated to yield the corresponding prenyl alcohols and subjected to reverse-phase TLC for chain length analysis. When the products of cytosol assays primed with DMAPP were compared, there was a small, but reproducible, increase in the synthesis of FOH (FPP before dephosphorylation) relative to GGOH (GGPP before dephosphorylation) in the preparation containing the recombinant protein. The assays primed with GPP showed a similar result. These data suggested that the recombinant Rv3398c may be a GGPP synthase capable of releasing detectable amounts of the intermediate, FPP, or that Rv3398c encodes a FPP synthase and an endogenous *M. smegmatis* GGPP synthase was depleting the product pool created by the recombinant enzyme. To differentiate between these two possibilities, isotopic trapping assays were conducted in which two allylic primers (GPP and *ω,E,E*-FPP, 50 μM each) were included in each assay mixture. Thus, if FPP was the true enzymatic product, radiolabeled FPP produced by the addition of [^{14}C]IPP to GPP would accumulate. The pool of cold *ω,E,E*-FPP would reduce the specific activity of the synthesized [^{14}C]FPP that was further elongated into [^{14}C]GGPP by any background *M. smegmatis* enzymes, thus reducing the incorporation of radiolabel into GGPP. Wild-type cytosol, simultaneously primed with GPP and FPP, primarily produced GGPP (**Fig. 2A**). Small amounts of FPP and heptaprenyl diphosphate were also synthesized. Figure 2B shows the results from an assay of Rv3398c recombinant cytosol primed with both GPP and FPP. In this case, the primary product is clearly FPP (or FOH after dephosphorylation).

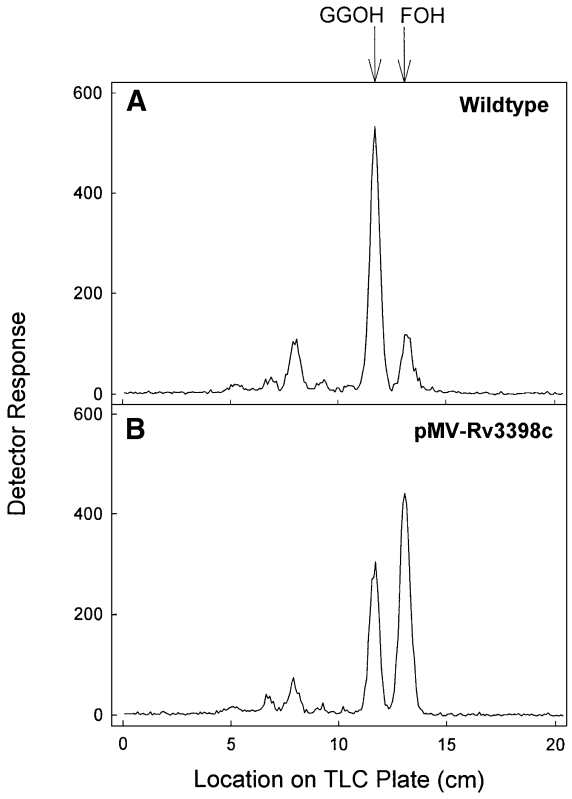

Figure 2. TLC analysis of [^{14}C]isopentenyl diphosphate ([^{14}C]IPP)-radiolabeled products synthesized in the presence of 50 μM geranyl diphosphate (GPP) and *ω,E,E*-FPP by cytosolic fractions prepared from wild-type *M. smegmatis* (A) or transformed *M. smegmatis* expressing pMV-Rv3398c (B). FPP synthase activity was assayed and the extracted isoprenyl diphosphates were dephosphorylated with potato acid phosphatase as described in Materials and Methods. Equal amounts of radioactivity were spotted on reverse-phase TLC plates, and the plates were developed in methanol-acetone (8:2, v/v). Products labeled with [^{14}C]IPP were visualized by a Bioscan System 200 Imaging Scanner. Standard polyprenols were located with anisaldehyde spray reagent. The migration of nonradioactive standards [geranylgeraniol (GGOH) and farnesol (FOH)] is indicated with arrows above the panels.

Stereochemical analysis of enzymatically synthesized FPP and GGPP

The FPP created by adding one molecule of [^{14}C]IPP to GPP can have two possible stereoconfigurations, *ω,E,E*-FPP and *ω,E,Z*-FPP. Similarly, the GGPP produced from the addition of one molecule of [^{14}C]IPP to *ω,E,E*-FPP can have the stereoconfigurations *ω,E,E,E*-GGPP or *ω,E,E,Z*-GGPP. The radiolabeled products (FPP and GGPP) produced in the double primer assays were dephosphorylated and separated by reverse-phase TLC. The radioactive compounds

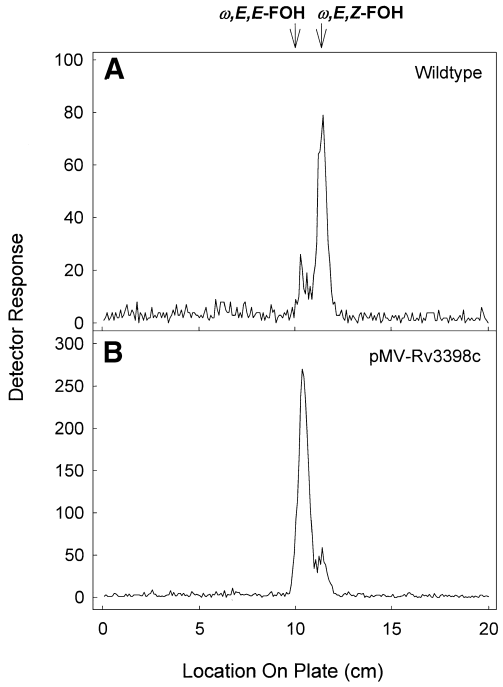

Figure 3. Stereochemical analysis of FPP enzymatically synthesized by the cytosolic fractions prepared from wild-type *M. smegmatis* (A) or transformed *M. smegmatis* expressing pMV-Rv3398c (B). Assay conditions are described in Materials and Methods. Assay products were dephosphorylated and subjected to reverse-phase TLC. The radiolabeled material corresponding to FOH was scraped from the reverse-phase TLC plates and extracted. The recovered FOH was analyzed by silica gel 60 TLC and developed in toluene-ethyl acetate (7:3, v/v). The location of the authentic standards (mixed isomers of FOH: *ω,E,E*-FOH and *ω,E,Z*-FOH) are shown above the panel.

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identified as FOH and GGOH were individually isolated and extracted from the reverse-phase gel and applied to silica gel 60 TLC plates. The majority of the FOH recovered from the wild-type strain migrated with standard *ω,E,Z*-FOH, which runs ahead of standard *ω,E,E*-FOH (**Fig. 3A**). In contrast, the majority of the FOH recovered from the Rv3398c-expressing strain comigrated with *ω,E,E*-FOH (Fig. 3B), representing at least a 10-fold increase in the specific activity of the *ω,E,E*-FPP synthase. The GGOH from both the wild-type and Rv3398c-expressor strains was in the *ω,E,E,E* stereoconfiguration (data not shown).

Expression and purification of affinity-tagged Rv3398c

Attempts at protein expression in *E. coli* or *M. smegmatis* using a mycobacterial expression vector (pVV16) that has previously been used successfully for convenient recombinant cloning and expression of *M. tuberculosis* genes fused to six histidine residues at the C-terminus of the protein (19, 21, 22) were unsuccessful. Construction of a new mycobacterial expression vector (pVV2) that encodes an in-frame N-terminal fusion with the protein of interest allowed the expression of Rv3398c with an N-terminal affinity tag. This fusion protein was well expressed and could be purified to near homogeneity (**Fig. 4**).

Substrate and product specificity of recombinant, histidine-tagged Rv3398c

The purified protein was assayed for [^{14}C]IPP incorporation into butanol-extractable material with five different allylic primers, DMAPP (C_5), GPP (C_{10}), neryl diphosphate (C_{10}), *ω,E,E*-FPP (C_{15}), and *ω,E,E,E*-GGPP (C_{20}). GPP was the only functional substrate (data not shown), as assays with the other allylic primers did not produce any detectable radioactive products. [^{14}C]IPP incorporation into butanol-extractable material in assays primed with GPP was linear for at least 20 min (data not shown). The product synthesized by the purified protein fraction was analyzed for chain length and stereochemistry by TLC and identified as *ω,E,E*-FPP, confirming the results of the isotope-trapping experiments (Fig. 3).

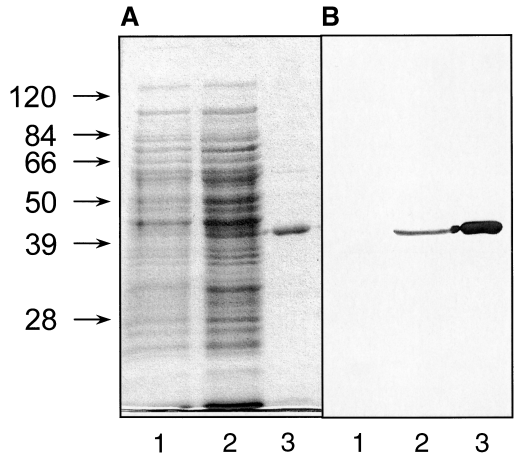

Figure 4. SDS-PAGE analysis of proteins from *M. smegmatis* transformed with pVV2-Rv3398c. Proteins from bacteria transformed with empty vector and bacteria transformed with pVV2-Rv3398c were loaded in lanes 1 and 2, respectively. Purified Rv3398c was loaded in lane 3. Proteins were visualized by staining with Coomassie Brilliant Blue R250 (A) or by Western blotting with mouse monoclonal anti-polyhistidine and anti-mouse IgG conjugated to alkaline phosphatase (B). Positions of molecular weight markers are shown at left.

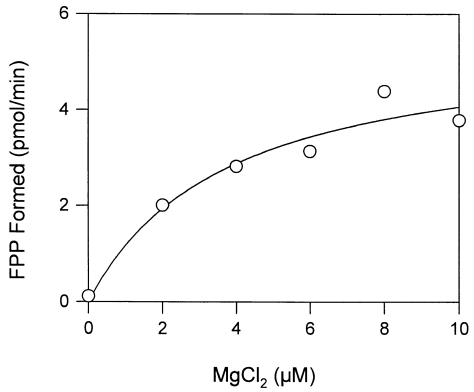

Figure 5. Divalent cation requirement for optimal Rv3398c activity. Purified enzyme was incubated with Bio-Rex 70 (sodium form, 200–400 mesh; Bio-Rad) on ice for 20 min to remove endogenous divalent cations. $MgCl_2$ was added to reaction mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2% Triton X-100, 30 μM [^{14}C]IPP, 100 μM GPP, and 13.6 pmol (5.3 μg) of recombinant protein in a final volume of 50 μl. Reactions were incubated for 10 min at 37°C, and the reaction was stopped by the addition of 1 ml of water saturated with NaCl. Radiolabeled products were extracted with butanol saturated with water and quantitated by liquid scintillation spectrometry.

Reaction requirements for Rv3398c

Rv3398c is absolutely dependent on the presence of divalent cations: the addition of 10 mM EDTA to the reaction mixture completely abolished enzymatic activity. Mg^{2+} supports optimal enzyme activity between 4 and 10 mM (**Fig. 5**); both Mn^{2+} and Fe^{2+} also supported activity, although at lower levels (**Table 1**). The enzyme was nearly inactive in the presence of Co^{2+}, and activity was undetectable in the presence of Ca^{2+}. The addition of 2.5 mM dithiothreitol increased the enzyme activity by 2-fold, and

TABLE 1. Effect of various divalent cations on Rv3398c activity

Divalent Cation	Concentration	
	1 mM	5 mM
	pmol/min	
Ca^{2+}	0.15	0.15
Co^{2+}	0.25	0.42
Fe^{2+}	0.24	1.6
Mn^{2+}	0.71	2.0
Zn^{2+}	0.14	0.08

Purified enzyme was incubated with Bio-Rex 70 (sodium form, 200–400 mesh; Bio-Rad) on ice for 20 min to remove endogenous divalent cations. $CaCl_2$, $Co(NO_3)_2$, $FeCl_2$, $MnCl_2$, or $ZnCl_2$ was added to reaction mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2% Triton X-100, 30 μM [^{14}C]isopentenyl diphosphate ([^{14}C]IPP), 100 μM geranyl diphosphate (GPP), and 13.6 pmol (5.3 μg) of recombinant protein in a final volume of 50 μl. Reactions were incubated for 10 min at 37°C, and the reaction was treated as described in Materials and Methods.

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the addition of Triton X-100 slightly increased the enzyme activity when added to a concentration of 0.2%. Enzyme activity was increased 1.5-fold at this concentration but was inhibited by higher concentrations of detergent. The enzyme was active over a broad range of pH values from 7 to 10, with optimal activity at pH 8.

Kinetic constants

The rate of *E,E,*-FPP synthesis was measured in the presence of 100 μM GPP and varying concentrations of [^{14}C]IPP (**Fig. 6A**), and a K_m^{IPP} value of 43 μM was calculated by nonlinear regression for the isoprenyl donor (**Table 2**). When the concentration of IPP was fixed at 30 μM and the concentration of GPP was varied, a K_m^{GPP} of 9.8 μM was calculated. As can be seen in Fig. 6B, there was no evidence of substrate inhibition at concentrations of GPP up to 1 mM.

DISCUSSION

We have demonstrated that open reading frame *Rv3398c* from the *M. tuberculosis* H37Rv genome encodes an isoprenyl diphosphate synthase that synthesizes *ω,E,E*-FPP from GPP and IPP. Rv3398c is unique in that it is the first reported eubacterial *ω,E,E*-FPP synthase that does not have four amino acids between the aspartate residues of the FARM and that has the features of the archaeal CLD region. All previously identified isoprenyl diphosphate synthases with these features are archaeal in origin and encode GGPP synthases. Thus, this enzyme is the first reported representative of a previously undescribed class of *ω,E,E*-FPP synthases.

TABLE 2. Calculated kinetic parameters for Rv3398c

Parameter	IPP	GPP
K_m (μM)	43	9.8
V_{max} (pmol min^{-1})	6.7	2.8
K_{cat} (min^{-1})	0.49	0.20
K_{cat}/K_m (min^{-1} M^{-1})	1.1×10^4	2.1×10^4

Farnesyl diphosphate synthase activity was assayed in mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2% Triton X-100, and 13.6 pmol (5.3 μg) of recombinant protein in a final volume of 50 μl. The concentration of [^{14}C]IPP was held at 30 μM and the concentration of the GPP was varied, or the concentration of GPP was held at 100 μM and the concentration of the [^{14}C]IPP was varied. Reactions were incubated for 10 min at 37°C, and the reaction was treated as described in Materials and Methods.

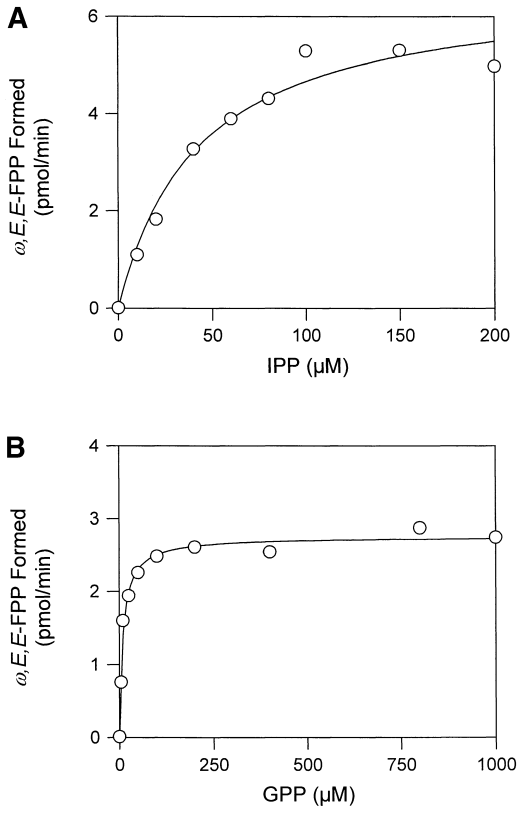

Figure 6. Effect of substrate concentration on Rv3398c activity. Enzyme activity was assayed in 50 μl mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2% Triton X-100, 5.3 μg of recombinant protein, and either 100 μM GPP and the indicated concentrations of [^{14}C]IPP (A) or 30 μM [^{14}C]IPP and the indicated concentrations of GPP (B). After incubating at 37°C for 10 min, the reaction was stopped by the addition of 1 ml of water saturated with NaCl. Radiolabeled products were extracted with butanol saturated with water and quantitated by liquid scintillation spectrometry.

The amino acid sequence of the mycobacterial *ω,E,E*-FPP synthase was aligned with 25 representative *ω,E,E*-FPP and GGPP synthases (5 archaeal, 10 bacterial, and 10 eukaryotic) and subjected to Clustal W analysis (**Fig. 7**). The type I eukaryotic and type II eubacterial *ω,E,E*-FPP synthases were separated into distinct branches as previously reported (1); however, the mycobacterial *ω,E,E*-FPP synthase was segregated and appears to be most closely related to type I GGPP synthases from *Archaea*. According to Wang and Ohnuma's evolutionary hypothesis (1), the characteristic change in the conversion of a type I GGPP synthase to a type II *ω,E,E*-FPP synthase appears to be a two amino acid insertion within the FARM. However, mutagenesis studies have shown that this insertion is not enough to confer FPP synthetic activity on a type I GGPP synthase (23), although a point mutation outside of the FARM has recently been shown to change product specificity in this class of enzyme (24). Therefore, CLD features exist in short-chain prenyl diphosphate synthases, which have not yet been well characterized; presumably, it is these characteristics that determine the product chain length of the enzyme encoded by *Rv3398c*.

To characterize the activity of Rv3398c, it was necessary to construct a mycobacterial vector that expresses recombinant proteins fused to an N-terminal histidine tag as opposed to a C-terminal tag. This is likely attributable to the fact that Rv3398c has positively charged side chains in two of the three C-terminal amino acid residues. Many other isoprenyl diphosphate synthases also have amino acids with positively charged side chains in the three C-terminal residues, which appear to help seal the active site during catalysis (3).

The enzyme has a broad pH optimum, an absolute requirement for divalent cation, and is optimally active in

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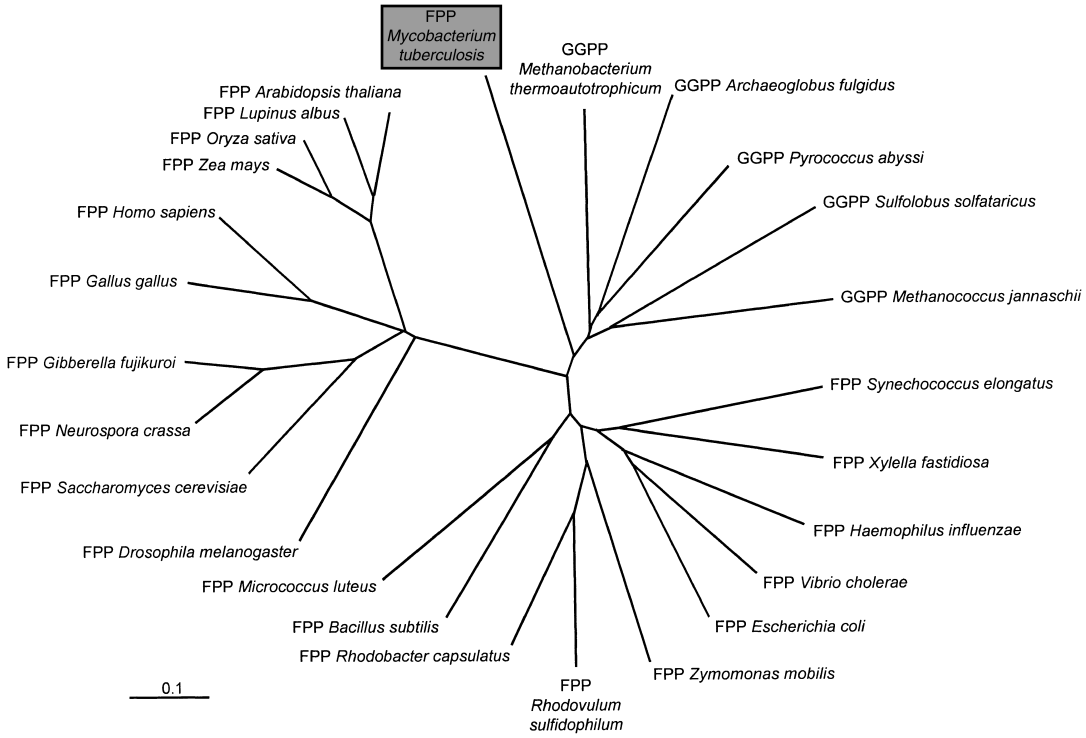

Figure 7. Unrooted dendrogram showing the results of Clustal W analysis of the mycobacterial *ω,E,E*-FPP synthase and 25 representative *ω,E,E*-FPP and *ω,E,E,E*-geranylgeranyl diphosphate synthases of archaeal, prokaryotic, and eukaryotic origin (accession numbers in parentheses). *Mycobacterium tuberculosis* (Q50727), *Drosophila melanogaster* (AAD27853), *Saccharomyces cerevisiae* (P08524), *Neurospora crassa* (Q92250), *Gibberella fujikuroi* (Q92235), *Zea mays* (P49353), *Oryza sativa* (T03687), *Lupinus albus* (P49352), *Arabidopsis thaliana* (Q09152), *Gallus gallus* (1065289), *Homo sapiens* (P14324), *Synechococcus elongatus* (BAA82614), *Xylella fastidiosa*, (AAF83471), *Escherichia coli* (P22939), *Vibrio cholerae* (AAF94052), *Haemophilus influenzae* (P45204), *Zymomonas mobilis* (AAF12843), *Rhodovulum sulfidophilum* (BAA96459), *Rhodobacter capsulatus* (BAA96458), *Bacillus subtilis* (P54383), *Micrococcus luteus* (O66126), *Sulfolobus solfataricus* (P95999), *Methanococcus jannaschii* (Q58270), *Methanobacterium thermoautotrophicum* (O26156), *Archaeglobus fulgidus* (F69535), and *Pyrococcus abyssi* (C75139). Bar = branch distance for 0.1 nucleotide substitutions per site.

the presence of dithiothreitol and Triton X-100. Other studies have shown that many *ω,E,E*-FPP synthases accept both DMAPP and GPP as allylic substrates (25–30). In cases in which *ω,E,E*-FPP is synthesized from IPP and DMAPP by a single enzyme, two condensations of IPP with the allylic substrate are catalyzed, releasing only small amounts of the GPP intermediate (31). Of the five potential allylic substrates tested here, including DMAPP, only GPP served as an acceptor for IPP. This specificity is similar to the specificity of the *ω,E,Z*-FPP synthase from *M. tuberculosis* that we characterized earlier (19) and indicates that a separate and, as yet unidentified, enzyme must exist in *M. tuberculosis* that synthesizes GPP. The K_m values for IPP and GPP reported here (43 and 9.8 μM, respectively) are similar to those reported for the *ω,E,Z*-FPP synthase (124 and 38 μM), suggesting that there is no likely preferential utilization of common substrates by one of the enzymes unless the substrates are compartmentalized.

High-density transposon mutagenesis experiments indicate that *Rv3398c* is transcribed, translated, and essential for *M. tuberculosis* growth (32). However, the function of the *ω,E,E*-FPP synthesized in this pathogen is not yet clear. The genome of *M. tuberculosis* H37Rv (13) may provide clues that help identify the function of the *ω,E,E*-FPP synthase. *Rv3398c* and the open reading frame adjacent (*Rv3397c*) appear to be in an operon-like structure, as the stop codon of *Rv3397c* and the start codon of *Rv3398c* are separated by only 28 bp. *Rv3397c* encodes a protein with significant homology to squalene and phytoene synthases, enzymes responsible for head-to-head condensations of the allylic diphosphates FPP and GGPP, respectively. The chromosomal organization suggests that *ω,E,E*-FPP synthesized by the enzyme encoded by *Rv3398c* may be used for squalene and possibly sterol synthesis in *M. tuberculosis*, a process that was reported in *M. smegmatis* (12). However, *Methylococcus capsulatus* and *Nannocystis exedens* are the only bacteria that have been clearly shown to synthesize sterols in amounts similar to those seen in eukaryotes (33), and the group that reported sterol synthesis in *M. smegmatis* has since indicated that the sterol isolated may have been derived from the culture medium (34).

In many bacteria, *ω,E,E*-FPP is an intermediate in the synthesis of *ω,E,E,polyZ*-undecaprenyl phosphate (35–37). However, *M. tuberculosis* synthesizes *ω,E,polyZ*-decaprenyl phosphate (38, 39), a structure that is not consistent with the use of *ω,E,E*-FPP as a synthetic intermediate. Alternatively, *ω,E,E*-FPP may be channeled into the synthesis of the octaprenyl diphosphate synthase previously reported in *M. tuberculosis* (11); this enzymatic activity, identified in *M. tuberculosis* membranes, was specifically stimulated by

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the addition of the allylic substrate *ω,E,E,E*-GGPP. In addition, it is likely that *ω,E,E*-FPP is used for menaqinone, heme O, and carotenoid synthesis, processes that have yet to be fully characterized in mycobacteria.

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