Identification of a Short (C₁₅) Chain Z-Isoprenyl Diphosphate Synthase and a Homologous Long (C₅₀) Chain Isoprenyl Diphosphate Synthase in Mycobacterium tuberculosis*  

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We report the cloning, overexpression, and partial characterization of two unique Z-isoprenyl diphosphate synthase homologs from Mycobacterium tuberculosis. The first enzyme, Rv1086, adds one isoprene unit to ω-E-geranyl diphosphate. The product, ω,E,Z-farnesyl diphosphate, is the putative substrate of the second enzyme, Rv2361c. This enzyme adds seven more isoprene units to ω,E,Z-farnesyl diphosphate and releases decaprenyl diphosphate. Both open reading frames were cloned from the M. tuberculosis H37Rv genome and overexpressed in M. smegmatis. Membrane and cytosol fractions from wild type and the two recombinant strains were assayed for [¹⁴C]isopentenyl diphosphate incorporation into isoprenyl diphosphates in the presence of various allylic isoprenyl diphosphate acceptors. Membrane fractions of recombinant cells overexpressing Rv2361c incubated with farnesyl diphosphate showed a 10-fold increase of [¹⁴C]isopentenyl diphosphate incorporation into decaprenyl diphosphate. Membrane fractions of recombinant cells overexpressing Rv1086 incubated with geranyl diphosphate showed a 5-fold increase of [¹⁴C]isopentenyl diphosphate incorporation into farnesyl diphosphate. Analysis of the stereochemistry revealed that all of the overexpressed farnesyl diphosphate was in the ω,E,Z-configuration. This is the first description of a short chain isoprenyl diphosphate synthase that generates products with Z-stereochemistry. Previously, all known short chain isoprenyl diphosphate synthases catalyze the synthesis of products with E-stereochemistry.

The isoprenoid compounds are chemically diverse, with over 23,000 compounds currently characterized (1). Representative members of this family of compounds (cholesterol, quinones, carotenoids, polyisoprene phosphates, and rubber) display diversity in structure as well as function. Polyprenyl phosphate (Pol-P)¹ is intimately involved in prokaryotic cell wall biosynthesis. In fact, evidence suggests that the rate of bacterial cell wall synthesis in vivo could be regulated by Pol-P levels (2–5). Eubacteria usually contain a single Pol-P molecule (C₃₅) composed of 11 isoprene units: ω,E,E,polyZ-undeacaprenyl-P.² Mycobacteria are exceptions to this rule. Takayama et al. demonstrated that M. smegmatis possesses two unique Pol-P: 1) a heptaprenyl phosphate (C₃₅) with four saturated, two E, and one Z double bond and 2) a decaprenyl phosphate (C₅₀) with one E and eight Z double bonds (6). In 1998, Wolucka and de Hoffman (7) isolated a form of heptaprenyl phosphate that contained four saturated and three Z-isoprene units from M. smegmatis. All of these Pol-P molecules were isolated as mannosyl-1-phosphorylpolyaprenols (Pol-P-Man), and probably have roles in mannan and arabinomannan synthesis (8). M. tuberculosis appears to be more typical of other eubacteria than M. smegmatis, since it appears to contain a single Pol-P molecule, decaprenyl phosphate, whose stereochemistry has not yet been determined (9).

We have implicated Pol-P-Man in the biosynthesis of mycobacterial lipomannan and lipoarabinomannan; Pol-P-Man is the direct donor of mannose to the phosphatidyl-D-myo-inositol oligomannosides to give rise to phosphatidyl-D-myo-inositol oligomannoside-containing lipomannan and lipoarabinomannan (10). In addition, heptaprenyl phosphate is found in the form of mycolyl-6-alpha-mannosyl-1-phosphorylheptaprenol and may function to carry mature mycolic acids across the plasma membrane (11). Arabinosyl-1-phosphoryldecaprenol is donor of the arabinofuranosyl residue in the arabinan of arabinogalactan, arabinomannan, and lipoarabinomannan (12). The disaccharide linker unit that bridges the arabinogalactan to the peptidoglycan is also formed while attached to Pol-P, which acts as the template for the synthesis of the entire mycolylarabinogalactan-linker unit complex (8). Pol-P (probably the decaprenyl phosphate) also has a role in mycobacterial peptidoglycan synthesis (5, 13). Despite its obvious importance, the genetics and biochemistry of Pol-P synthesis have not been investigated in mycobacteria.

All known isoprenoids share a common biosynthetic mechanism beginning with the condensation of two five carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate, to form geranyl diphosphate (GPP, C₁₀) (14). This and subsequent additions of IPP to the growing allylic diphosphate (to form farnesyl diphosphate (FPF, C₁₅) and geranylgeranyl diphosphate (GGPP, C₂₀), etc.) are catalyzed by a family of enzymes known as isoprenyl diphosphate synthases. The sequential addition of IPP to allylic diphosphate precursors continues until a physiologically relevant chain length is reached. At this point, the molecule can be dephosphorylated to form Pol-P.² The stereochrmical configuration of the isoprene units are listed starting at the ω-end of the molecule.

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¹ The abbreviations used are: Pol-P, polyprenyl phosphate; DecaPP, decaprenyl diphosphate; FPP, farnesyl diphosphate, no stereochemistry assigned; IPP, isopentenyl diphosphate; ω,E-GPP, ω,E-geranyl diphosphate; ω,E,Z-FPP, ω,E,Z-farnesyl diphosphate; ω,E,E,FPP, ω,E,E-farnesyl diphosphate; Pol-P-Man, mannosyl-1-phosphorylheptaprenol; Z-FPPS, ω,E,Z-farnesyl diphosphate synthase; MOPS, 4-morpholinepropanesulfonic acid.

² The stereochrmical configuration of the isoprene units are listed starting at the ω-end of the molecule.
Only a small fraction of the enzymes involved in isoprenoid chain elongation have been studied, and genetic information is available for only a subset of these (1). Amino acid alignments of these enzymes generated by Chen et al. in 1994 (15) and again by Kellogg and Poulter in 1997 (16) have defined five conserved regions, including two aspartic acid-rich (DD/XXD)D motifs. However, the resulting consensus sequences have to date been useful in identifying only those enzymes catalyzing the formation of E double bonds. Enzymes responsible for catalyzing the formation of Z double bonds are believed to be of a different family. Recently, a publication described the molecular cloning, expression, and purification of undecaprenyl diphosphate synthase, an enzyme from Micrococcus luteus that catalyzes Z-polyisoprenyl chain elongation of the allylic substrate ω,E,E-FPP (17), the first such enzyme to have its amino acid sequence determined. As anticipated, it had no homology to any of the E-isoprenyl diphosphate synthases. Apfel et al. (18) published an alignment of 28 putative undecaprenyl diphosphate synthase homologs and demonstrated long chain prenyltransferase activity for three of them (Escherichia coli, Haemophilus influenzae, Streptococcus pneumoniae). Also, the Z-isoprenyl diphosphate synthase (dolichol synthase) from Saccharomyces cerevisiae has been identified (19, 20).

The determination of the complete genome sequence for Mycobacterium tuberculosis H37Rv has allowed for systematic exploration of gene function in this organism (21). We have identified two open reading frames in the M. tuberculosis H37Rv genome, Rv1086 and Rv2361c, whose predicted protein sequence determined. As anticipated, it had no homology to any of the E-isoprenyl diphosphate synthases. Apfel et al. (18) published an alignment of 28 putative undecaprenyl diphosphate synthase homologs and demonstrated long chain prenyltransferase activity for three of them (Escherichia coli, Haemophilus influenzae, Streptococcus pneumoniae). Also, the Z-isoprenyl diphosphate synthase (dolichol synthase) from Saccharomyces cerevisiae has been identified (19, 20).

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RESULTS

Recombinants Rv1086 and Rv2361c Increase [14C]IPP Incorporation into Butanol-extractable Material—Rv1086 and Rv2361c were cloned into pVV16. In this mycobacterial specific expression vector, the cloned genes are constitutively expressed under the control of the heat shock promoter HSP60. Protein (membrane or cytosol) from wild type, empty vector or recombinant strains was assayed for [14C]IPP incorporation into butanol-extractable material in the presence of \( \omega_E \)-GPP or \( \omega_E \)-E-FPP as the reaction primer. The presence of pVV16 (empty vector) in bacteria and the required kanamycin in the growth medium had no effect on the expression of isoprenyl diphosphate synthases (data not shown). Both Rv1086 and Rv2361c cytosolic assays primed with \( \omega_E \)-GPP showed an increase in [14C]IPP incorporation into butanol-extractable material when compared with the wild type cytosolic assays (Table I). However, only the Rv2361c cytosolic assay primed with \( \omega_E \)-E-FPP showed an increase in [14C]IPP incorporation. Assays of membrane protein revealed similar results (Table I). The Rv2361c recombinant membrane protein was able to utilize both primers more effectively than wild type membrane protein. The Rv1086 recombinant membrane protein was able to utilize more [14C]IPP than wild type membrane protein when primed with \( \omega_E \)-GPP but not when primed with \( \omega_E \)-E-FPP. Protein fractions derived from the strains expressing Rv1086 or Rv2361c had increased isoprenyl diphosphate synthase activity when compared with the corresponding wild type protein fractions.

TLC Analysis Reveals That the Rv1086 and Rv2361c Recombinants Have Increased [14C]IPP Incorporation into Farnesyl Diphosphate and Decaprenyl Diphosphate, Respectively—The products of the above assays were subjected to enzymatic dephosphorylation for analysis of chain length by TLC. Butanol-extracted reaction products were dephosphorylated, and equal amounts of radioactivity were loaded onto reverse-phase TLC plates. Figs. 1 and 2 show the migration of [14C]IPP-labeled products from the cytosol and membrane reactions, respectively. Wild type cytosol primed with \( \omega_E \)-GPP (Fig. 1A) primarily produces geranylgeranyl diphosphate (specific activity = 173 pmol/mg/min). The smaller amounts of FPP (specific activity = 69 pmol/mg/min) and heptaprenyl diphosphate (specific activity = 27 pmol/mg/min) may be due to imperfect fractionation of membrane and cytosolic proteins as the products are seen at approximately 2–3-fold higher concentration in the membrane assays (compare Figs. 1A and 2A). Wild type cytosol when primed with \( \omega_E \)-E-FPP (Fig. 1B), once again primarily produced geranylgeranyl diphosphate (specific activity = 169 pmol/mg/min). Rv2361c cytosolic assays primed with \( \omega_E \)-GPP and \( \omega_E \)-E-FPP (Fig. 2, C and D) showed an increase in the synthesis of decaprenyl diphosphate (specific activity = 141 and 126 pmol/mg/min, respectively), an activity that was almost undetectable in wild type cytosol. Products corresponding to the calculated migrations of octaprenol and nonaprenol are also present. Rv1086 cytosolic assays primed with \( \omega_E \)-GPP (Fig. 1E) showed a 10-fold increase of [14C]IPP incorporation into FPP (specific activity = 714 pmol/mg/min) versus wild type (specific activity = 69 pmol/mg/min), whereas Rv1086 cytosolic assays primed with \( \omega_E \)-E-FPP (Fig. 1F) showed 3-fold decrease in FPP synthesis (but had approximately equal heptaprenyl diphosphate synthesis) when compared with the wild type.

### Table I

Incorporation of [14C]IPP into allylic diphosphates catalyzed by cytosolic or membrane fractions prepared from wild type M. smegmatis, or recombinant M. smegmatis

| Aliphatic diphosphate | Cytosolic activity | Membrane activity |
|-----------------------|--------------------|-------------------|
| \( \omega_E \)-GPP | WT Rv1086 Rv2361c | WT Rv1086 Rv2361c |
| pmol/mg/min | | |
| \( \omega_E \)-E-FPP | 298 296 614 | 432 966 1108 |
| | 245 145 476 | 260 258 487 |

...
When the products from the wild type membrane assays primed with \( \omega_E\)-GPP (Fig. 2A) are compared with the recombinant membrane assays primed with \( \omega_E\)-GPP (Fig. 2, C and E), there is a significant increase in \([\text{14C}]\text{IPP}\) incorporation into decaprenyl diphosphate caused by the expression of Rv2361c (specific activity = 443 pmol/mg/min) into FPP caused by the expression of Rv1086 (specific activity = 701 pmol/mg/min). In the membrane assays primed with \( \omega_E,E\)-FPP (Fig. 2, B, D, and F), there is a 10-fold increase of \([\text{14C}]\text{IPP}\) incorporation into decaprenyl diphosphate by the Rv2361c recombinant (specific activity = 205 pmol/mg/min) compared with the corresponding wild type assay. As expected from the results in Table I, the Rv1086 membrane assay primed with \( \omega_E,E\)-FPP did not reveal any increased incorporation when compared with the corresponding wild type assay.

**Rv1086 Catalyzes Synthesis of \( \omega_E,Z\)-Farnesyl Diphosphate**—The farnesyl diphosphate created by adding one molecule of \([\text{14C}]\text{IPP}\) to \( \omega_E\)-GPP can have two possible stereochemistries, \( \omega_E,E\)-FPP or \( \omega_E,Z\)-FPP. It is possible to separate these stereoisomers by silica gel TLC (Fig. 3, lane 2). The farnesol produced from enzymatically dephosphorylating the products of the Rv1086 membrane assay primed with \( \omega_E\)-GPP (Fig. 2E) was recovered from reverse-phase TLC plate and loaded onto silica gel 60 TLC plates. The recovered farnesol migrated along with standard cold \( \omega_E,Z\)-farnesol, which runs ahead of cold standard \( \omega_E,E\)-farnesol (Fig. 3, lanes 2 and 3). The farnesol produced from enzymatically dephosphorylating the products of the Rv1086 cytosolic assay primed with \( \omega_E\)-GPP was also in the \( \omega_E,Z\)-configuration (data not shown).

**DISCUSSION**

The *M. tuberculosis* open reading frame Rv2361c encodes a \( \omega_E,Z\)-farnesyl diphosphate synthase (Z-FPPS). This is the first report of an amino acid sequence for a short chain \( Z \)-isoprenyl diphosphate synthase. Previously, the family of \( Z \)-isoprenyl diphosphate synthases contained only undecaprenyl diphosphate synthases, dolichol synthases, and rubber synthase.

Based on the relative lengthy nature of their products (C\(_{55}\) or greater) and the common \( Z \)-stereochemistry catalyzed, members of the \( Z \)-isoprenyl diphosphate synthase family became synonymous with “long chain” isoprenyl diphosphate synthases. It is now clear that short chain \( Z \)-isoprenyl diphosphate synthases exist. However, with the amount of amino acid sequence information available at this time, short chain \( Z \)-isoprenyl diphosphate synthases remain indistinguishable from the long chain \( Z \)-isoprenyl diphosphate synthase in homologous types. The fact that the cloned \( Z \)-FPPS activity was equally distributed between the cytosolic and the membrane fractions confirms our earlier observations that *M. tuberculosis* cytosolic and membrane fractions contain nearly equal amounts of \( Z \)-FPPS activity, whereas the \( Z \)-FPPS activity of *M. smegmatis* was preferentially localized to the membrane fraction.

The *M. tuberculosis* open reading frame Rv2361c encodes a decaprenyl diphosphate (DecaPP) synthase. If the *M. tuberculosis* DecaPP synthase produces a product stereochemically identical to the DecaPP synthase from *M. smegmatis*, then its presumely allylic diphosphate substrate in vitro would be \( \omega_E,Z\)-FPP, and each molecule of IPP would be added with \( Z \)-stereoconfiguration to yield \( \omega_E\text{poly}Z\text{-decaprenyl diphosphate} \) (6). Our assays showed that Rv2936c was able to use the allylic primers \( \omega_E\)-GPP and \( \omega_E,E\)-FPP. \( \omega_E\)-GPP may not be used directly for DecaPP synthesis but instead may be used for \( \omega_E,Z\)-FPP synthesis by the background wild type *M. smegmatis* enzymes. \( \omega_E,Z\)-FPP is then used for DecaPP synthesis. This would explain the decrease in the amount of FPP seen in the Rv2361c membrane assay (Fig. 2C) when compared with the wild type membrane assay (Fig. 2A). On the other hand, \( \omega_E,E\)-FPP was also a functional substrate for DecaPP synthesis. Precedence for this lack of absolute substrate specificity has been demonstrated in vitro with other isoprenyl diphosphate synthases (24–26). The stereochemistry of each isoprene addition by DecaPP synthase has not been established. However, based on amino acid sequence homology between Rv2361c and the known \( Z \)-isoprenyl diphosphate synthases, it is fair to assume that each isoprene addition would be in the \( Z \)-stereoconfiguration.

The first \( Z \)-isoprenyl diphosphate synthase to have its amino acid sequence reported, undecaprenyl diphosphate synthase
had no homology to the E-isoprenyl diphosphate synthases (15, 16). It did however contain an aspartate rich DD(XX)2D motif reminiscent of the E-isoprenyl diphosphate synthases. Shimizu et al. (17) suggested that this motif may represent the diphosphate binding site. The signature motif (Fig. 4, amino acids 168–172 in MICLU) is not conserved among the M. tuberculosis Z-isoprenyl diphosphate synthases described here or any of the other known Z-isoprenyl diphosphate synthases. Therefore, its occurrence in M. luteus undecaprenyl diphosphate synthase is probably coincidental and not related to enzymatic activity.

We have generated an alignment of M. tuberculosis open reading frames Rv2361c and Rv1086 of M. tuberculosis H37Rv genome sequence with known Z-isoprenyl diphosphate synthases. Conserved amino acids are highlighted in black. Conserved regions are indicated with bars A–D. The DD(XX)2D motif in MICLU (amino acids 168–172, gray highlight) does not exist in other homologs. MICLU, M. luteus undecaprenyl diphosphate synthase, Swiss-Prot O82827; ECOLI, E. coli undecaprenyl diphosphate synthase, Swiss-Prot Q47675; HAEIN, H. influenzae undecaprenyl diphosphate synthase, Swiss-Prot P44938; STRPN, S. pneumoniae undecaprenyl diphosphate synthase (18); YEAST, S. cerevisiae dehydrodolichyl diphosphate synthetase, Swiss-Prot P35196. Alignment was generated by Multalin and edited in GeneDoc.

hypothetically aid in binding of the diphosphate moiety in the active site. Region B also contains conserved amino acids with large aromatic side chains such as tryptophan and phenylalanine (Fig. 4, amino acid positions 121 and 126). However, the Rv1086 (Z-FPPS) sequence contains leucine at both of these positions (the relevance of this particular observation is unknown).

The amino acid sequences of Rv1086 and Rv2361c were also analyzed for secondary structure using various computer programs (TMpred, TMAP, TopPred2, and SOSUI). None of the programs reported a membrane-spanning region in Rv1086; however, TMpred and TMAP both predicted a single membrane-spanning region (amino acids 92–120) in Rv2361c. The preferred model from the TMpred report suggested that the amino terminus of the protein exists outside the membrane, amino acids 92–120 span the membrane, and the remaining carboxyl terminus of the protein is on the interior of the membrane. This model is consistent with our observations in that DecaPP synthase activity is found in the membrane.

7 Tmpred can be found on the World Wide Web.
8 TMAP can be found on the World Wide Web.
9 TopPred2 can be found on the World Wide Web.
10 SOSUI can be found on the World Wide Web.
fraction of M. tuberculosis H37Rv, and the subcellular location of Rv1086 is more ambiguous as activity can be found in both the cytosolic and membrane fractions. Until more information about the active sites and crystal structures of Z-isoprenyl diphosphate synthases is gathered, it will not be possible to predict the chain length of the products of these enzymes based on amino acid sequence alone. Recently, crystallization and preliminary x-ray diffraction studies have been reported for the M. luteus undecaprenyl diphosphate synthase (27). It would be of great interest to determine the quaternary structures of the mycobacterial enzymes, since they could reveal structural requirements for chain length determination. We have initiated purification of these enzymes.

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