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DISSERTATION

ISOPRENOID CHAIN ELONGATION IN *MYCOBACTERIUM TUBERCULOSIS*

Submitted by

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Department of Microbiology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2000

COLORADO STATE UNIVERSITY

DECEMBER 6, 2000

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARK CONRAD SCHULBACH ENTITLED ISOPRENOID CHAIN ELONGATION IN MYCOBACTERIUM TUBERCULOSIS BE ACCEPTED AS FULFULLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ISOPRENOID CHAIN ELONGATION IN *MYCOBACTERIUM TUBERCULOSIS*

Isoprenyl diphosphate synthases catalyze the sequential addition of isopentenyl diphosphate to an allylic diphosphate acceptor until a physiologically relevant chain length has been reached. Seven open reading frames from the *Mycobacterium tuberculosis* H37Rv genome were identified as putative isoprenyl diphosphate synthases based on amino acid sequence similarities to known isoprenyl diphosphate synthases. In an attempt to map the pathway for the synthesis of decaprenyl phosphate, these open reading frames were cloned and overexpressed in *M. smegmatis*. Protein derived from wildtype or recombinant *M. smegmatis* was assayed for isoprenyl diphosphate synthase activity. This strategy yielded the identification of two unique *Z*-isoprenyl diphosphate synthases (corresponding to open reading frames Rv1086 and Rv2361c) and one *E*-isoprenyl diphosphate synthase (corresponding to open reading frame

Rv3398c). Rv1086 was further purified to homogeneity and tested for activity in the presence of inhibitors.

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DEDICATION

This dissertation would not be complete without mentioning the individuals who have inspired me to pursue this degree. First, I must mention my family (Mom, Dad, Julie) for their patience and love. They have given me confidence, freedom and inspiration to pursue dreams. Sherwin Kamkar, my best friend, for time spent expanding our young minds, for sharing his creative energy, challenging me and encouraging me to do my best. David Sullivan for showing me how to read books, Jason Zell, a role model in every aspect of life. Brannon Daly for being a life-long friend. Jim Ranta a friend and coach for life. Brice Daly, Travis Robson, John Riess- new friendships that I will cherish like the old. Saving the best for last, my girlfriend, Maile Fink for her love throughout the years. Oh yeah, and Robert Pirsig for writing books, Ed Vedder and Brad Nowell for singing songs.

"...this wind will blow, clearing the fog and producing the largest and steepest wave ever known..."

-mcs

TABLE OF CONTENTS

LIST OF TABLES AND SCHEMES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALA	D-alanine
D-ALA-D-ALA	D-alanyl-D-alanine
D-GLU	D-isoglutamate
DAP	meso-diaminopimelic acid
DMAPP	dimethylallyl diphosphate
DOTS	directly observed therapy short course
FARM	first aspartate rich motif
FPP	farnesyl diphosphate
GDP	guanidine diphosphate
GGPP	geranylgeranyl diphosphate
GlcNAc	N-acetylglucosamine
GPP	geranyl diphosphate
HMG-CoA	3-hydroxyl-3-methylglutaryl-CoA
IPP	isopentenyl diphosphate
L-ALA	L-alanine
LAM	lipoarabinomannan
MDR	multiple drug resistant
MurNAc	N-acetylmuramic acid
NPP	neryl diphosphate
PEP	phosphoenolpyruvate
PI	phosphatidylinositol
PIM_X	phosphatidylinositol $(mannoside)_x$

Pol-P	polyprenyl phosphate
Pol-P-Man	mannosyl-1-phosphoryl-polyprenol
Pol-P-P	polyprenyl diphosphate
SARM	second aspartate rich motif
UDP	uridine diphosphate
WHO	World Health Organization

Chapter 1.

Literature Review Part I- *Mycobacterium tuberculosis*

A. Historical Perspective

Paleopathogical evidence, archaeological finds and ancient writings suggest that *Mycobacterium tuberculosis* has infected man for more than 3000 years[1,2]. However, the notion that tuberculosis is a contagious disease caused by a microorganism was not definitively established until Robert Koch, in 1882, presented techniques that had allowed him to stain and visualize pure cultures of *M. tuberculosis*, the causative agent of tuberculosis[3].

Prior to Koch's revolutionary studies, some people considered tuberculosis to be caused by evil spirits, odors from foul sewage or swamplands, or spiritual corruption within the body. There was an abundance of treatment options for tuberculosis-sufferers in this era, however in terms of actual medicinal benefit, these options were painfully limited. Some patients were advised by their doctors to drink potions containing savory ingredients like garlic and dog fat, inhale smoke from burning cow dung or place seaweed under their beds[4]. Other obscure, albeit traditional treatments included hypnosis, purging, and blood-letting.

Notions of a physical contagion, suggestive of a germ-disease theory, have existed in literature since the the 5th century BC. However, it was Koch's landmark experiments that put to rest the theory that pulmonary tuberculosis arose spontaneously, and provided the groundwork for the development of the science of microbiology. From this point on, scientists and doctors have faced the arduous task of trying to eliminate a stubborn enemy, *M. tuberculosis*.

As the cause and mode of transmission was established for tuberculosis, interest became focused on preventative measures. Public education and awareness campaigns explained the benefits of covering the mouth when coughing or sneezing. These campaigns also encouraged the public to attend tuberculosis diagnostic clinics. Perhaps the most important control measure instigated to stop the spread of tuberculosis was the establishment of sanatoria. Sanatoria provided tuberculosis-specific in-patient care. The patients received a nutritious diet, fresh air, sunlight, spring water and bed rest. Those patients whose infections became latent and non-infectious were allowed to return home, whereas those patients who remained ill and contagious stayed within the confines of the sanatoria until death.

Chemotherapy for *M. tuberculosis* infections became available in 1944 when streptomycin, a molecule purified from

Streptomyces griseus, was administered to a critically ill tuberculosis patient[5]. In the following months, the patient made an impressive recovery and the tubercle bacilli were cleared from her sputum. Though streptomycin displayed some toxic side effects, the patient completely recovered from tuberculosis. Soon after, it was discovered that streptomycin monotherapy often generated mutant bacilli that were no longer sensitive to streptomycin[6]. Fortunately, over the next 20 years more anti-tuberculosis drugs were identified (p-aminosalicylic acid, isoniazid, pyrazinamide, cycloserine, ethambutol and rifampicin) and it was demonstrated that the drug resistance problem could be avoided by administering a combination of two or three drugs[7].

Antibiotic therapy revolutionized the treatment of tuberculosis as patients made complete and rapid recoveries[4]. By the mid-1960s and early 1970s, the majority of patients were treated at home and the sanatorial beds soon emptied. Between 1953 and 1984, the annual number of tuberculosis cases in the United States dropped from 84,304 to 22,225[8].

B. Tuberculosis Today

The era of successful tuberculosis treatment was rather short-lived. The global tuberculosis pandemic is once again displaying growing trends of morbidity and mortality[8]. The resurgence of tuberculosis has been attributed to two main

factors: 1) the increased incidence of people infected with the human immunodeficiency virus, whose immunocompromised condition increases susceptibility to tuberculosis infection, and 2) an increase in number of multiple drug resistant (MDR) tuberculosis cases, a phenomenon which is largely a consequence of the dismantled or poorly managed tuberculosis control programs[8].

MDR-tuberculosis is defined by those strains that are resistant to two or more frontline anti-tuberculosis drugs[9]. To rid the patient of these bacilli, more expensive second-line drugs must be administered and hospitals must take special precautions to prevent the spread of the resistant bacilli. The World Health Organization (WHO) reported that outbreaks of MDR tuberculosis has cost New York City hundreds of lives and more than $1 billion (US)[9]. The WHO further warns that if countries do not act quickly to strengthen their tuberculosis control programs, MDR tuberculosis will continue to emerge in other parts of the world, creating a morbid and economic crisis.

The WHO has developed the tuberculosis control program, Directly Observed Therapy Short-Course (DOTS)[9]. Under the DOTS program, trained nurses and health program volunteers interact with the patient to ensure the correct regimen of drugs is administered for six months, the time required to

complete the therapy. The DOTS program is very economical and has been extremely successful. In some countries, a six-month supply of isoniazid, ethionaminde, rifapicin, and ethambutol (the DOTS drug regimen) is only $11.00(US) per patient and the program has reported cure rates up to 95%[9]. Perhaps more importantly, the combined multi-drug regimen in the DOTS program prevents the development of MDR tuberculosis. However, the DOTS program is not yet available to all persons suffering from tuberculosis. In 1998, only about 21 percent of all tuberculosis patients were treated with the DOTS strategy[9].

The increasing number of MDR tuberculosis infections within the human population necessitates the development of new anti-mycobacterial agents. Fortunately, advances in mycobacterial research has provided access to a large amount of genetic, physiological and biochemical information about *M. tuberculosis*[10], information that has the potential to contribute to the rational development of new anti-mycobacterial drugs[10].

C. The Cell Wall

Introduction- There has been considerable interest in elucidating a detailed description of the mycobacterial cell wall[11]. The major component of the mycobacterial cell

envelope is the insoluble, covalently bound matrix composed of peptidoglycan, arabinogalactan and mycolic acids[12;13] (Figure

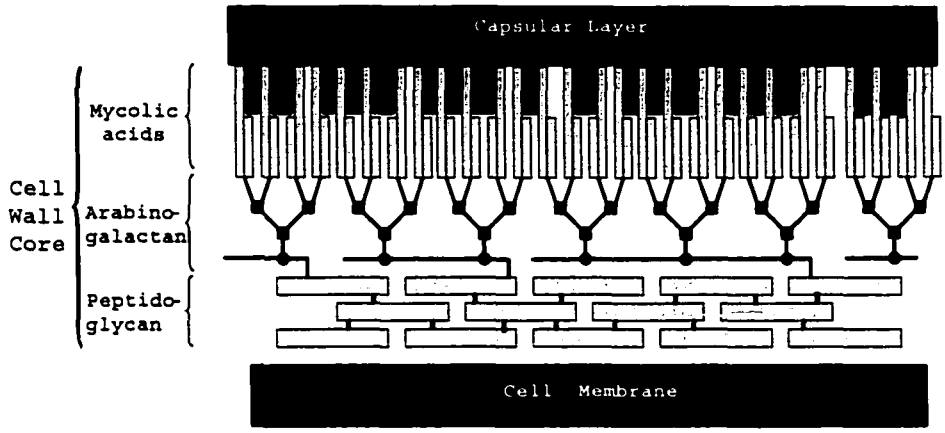

Figure 1.1- A schematic representing the major structures of the mycobacterial cell wall. The peptidoglycan, arabinogalactan and mycolic acid layers make up the covalently bound cell wall core.

1.1). Intimately associated with the mycolic acid layer is a capsular layer composed of numerous amphiphilic glycolipids[12] and surface-exposed polysaccharides[14;15]. Lipoarabinomannan (LAM) is a heterogeneous population of phosphatidylinositol anchored lipoglycans which are non-covalently associated with the cell envelope[16]. It is generally believed that the acyl portions of LAM are anchored in the cytoplasmic membrane and the carbohydrate chains extend outward towards the extracellular milleu[12].

Many cell wall components have had their structures resolved. Now research emphasis is shifting towards understanding their biosynthesis. The remaining goals of this chapter are two-

fold. First, the reader will be introduced to some of the key components of the mycobacterial cell wall. Second, the reader will review the biosynthesis of those components, paying particular attention to the roles involving polyprenyl phosphate (Pol-P).

Structure Of The Peptidoglycan-Arabinogalactan-Mycolic Acid Complex- Mycobacterial peptidoglycan (Figure 1.2) is a polymer of alternating N-glycolylmuramic acid -(β-1,4)- N-acetylglucosamine residues[13]. Attached to the lactate moiety

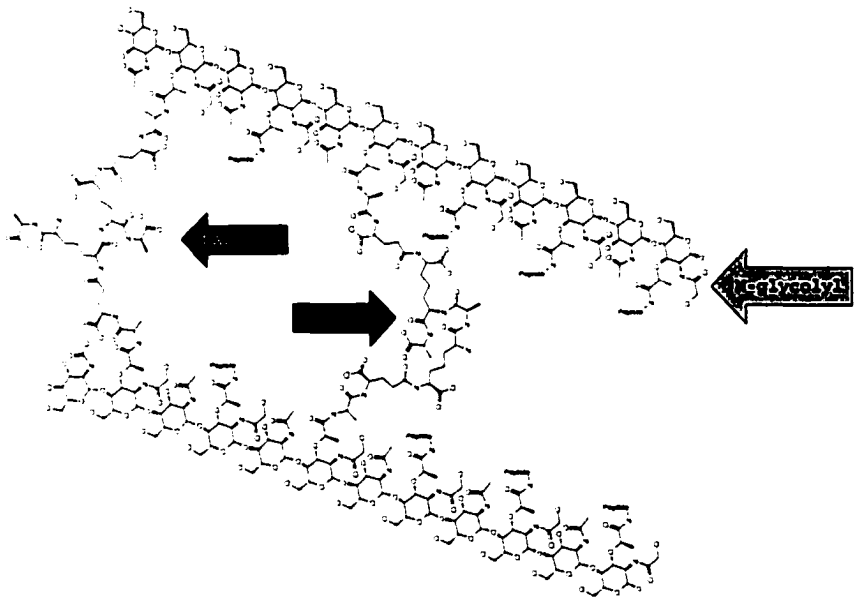

Figure 1.2- The structure of peptidoglycan from *M. tuberculosis*. Two strands of glycan are crosslinked with a DAP-DAP linkage (left) and DAP-ALA linkage (right). The muramic acid residues are N-glycoylated, not N-acetylated.

of N-glycolylmuramic acid is the tetrapeptide: L-alanine-D-isoglutamate-meso-diaminopimelic acid (DAP)-D-alanine (ALA). The muramyl peptides are crosslinked from one glycan chain to

another to form the stable murein complex. Both DAP-DAP crosslinks and DAP-ALA crosslinks have been identified in *Mycobacterium* spp[17].

The cell wall complex also contains arabinogalactan (Figure 1.3), which is covalently bound to C_6 of N-glycolylmuramic acid through an α-L-rhamnopyranose-(1-3)-α-D-GlcNAc-1-phosphate linker region[18]. The galactan portion is composed of alternating 5- and 6- linked β-D-galactofuranosyl residues[19]. Some of the 6-linked galactose residues are linked to an arabinan chain via carbon-5. The arabinan chains are composed of linear 5-linked α-D-arabinofuranosyl units with branching introduced by 3,5-α-D-arabinofuranosyl residues. Finally, the non-reducing termini of the arabinan chains are terminated with pentaarabinosyl motifs, [β-D-

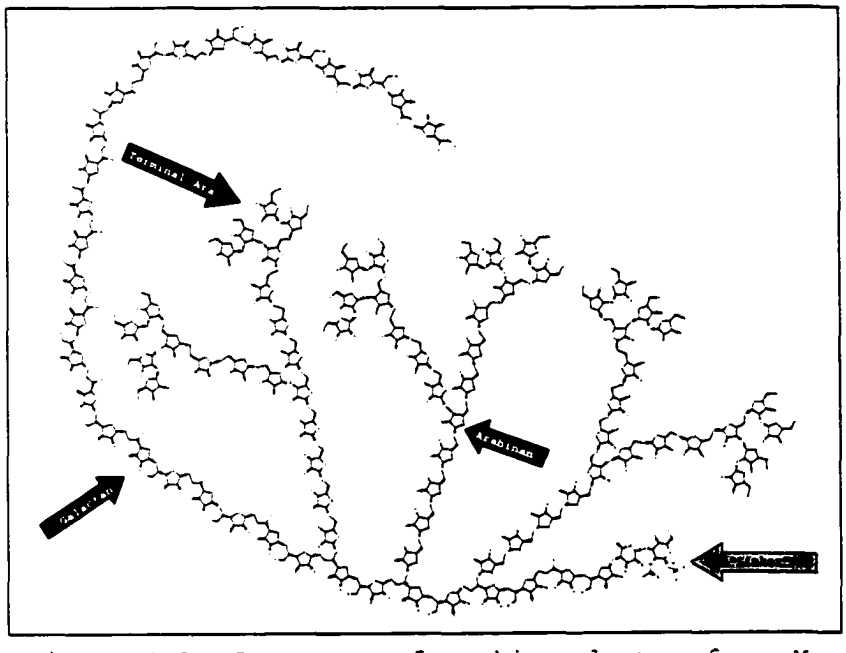

Figure 1.3- Structure of arabinogalactan from *M. tuberculosis*. See text for details.

arabinofuranosyl- (1-2)- α - D -arabinofuranosyl]$_2$ - 3,5 - α - D - arabinofuranosyl - (1-5) -α-D-arabinofuranose. About two-thirds of the pentaarabinosyl motifs are esterified with mycolic acids.

Mycolic acids are high-molecular-weight, α-branched, β-hydroxy fatty acids[20]. The α branch of mycolic acid is typically a C_{24}-C_{26} fatty acid. In contrast, the mero-aldehyde portion displays quite a bit of heterogeneity (keto groups, methoxy groups, cyclopropane rings). The figure below shows three representative mycolic acids found in *M. tuberculosis*.

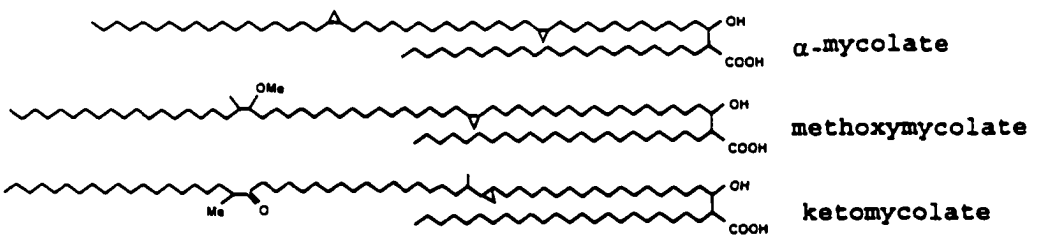

Figure 1.4- Representative mycolic acids from *M. tuberculosis*.

The Capsular Glycolipids and LAM- The non-covalently associated capsular glycolipids of *M. tuberculosis* (Figure 1.5) include the trehalose-based glycolipids: cord factor, sulfolipids and 2,3-di-O-acyltrehaloses[12;21]. Several species of trehalose esters have been identified in *M. tuberculosis*. The mycolipenic and mycolipanolic substituted forms are only

found in virulent strains of *M. tuberculosis*. Lipooligosaccharides and phenolic glycolipids (the well studied glycolipids of other species of mycobacteria) are not found in virulent strains of *M. tuberculosis*[20;21].

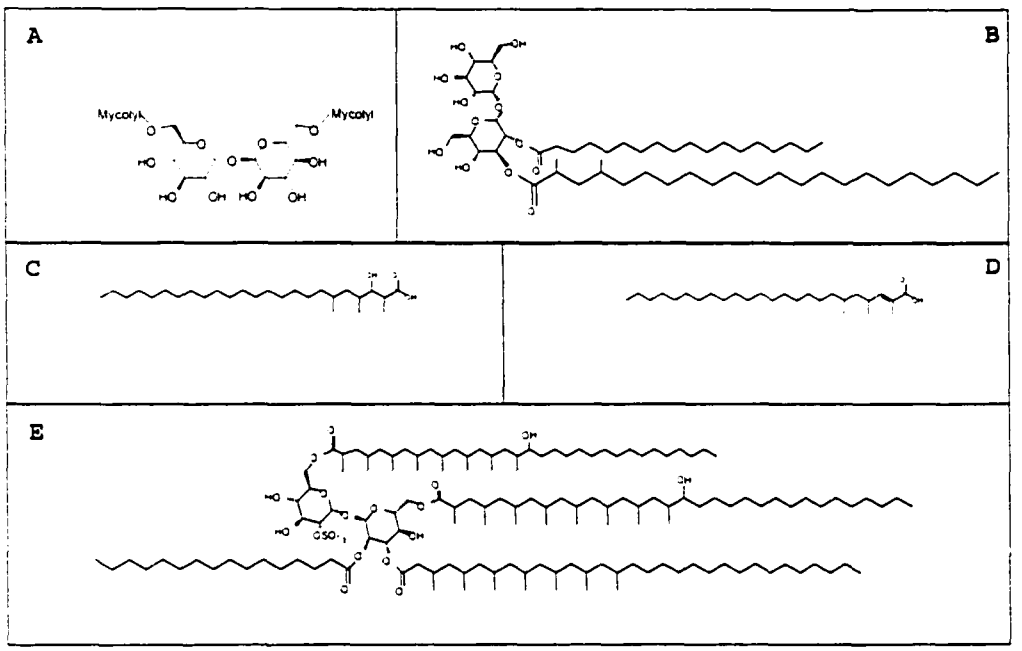

Figure 1.5- Capsular glycolipids of *M. tuberculosis*. Panel A- Trehalose 6,6'-dimycolate (Cord Factor). Panel B- Trehalose dervived glycolipid: 2,3-di-O-acyltrehalose. Other trehalose derived glycolipids from virulent *M. tuberculosis* contain mycolipanolic acid (Panel C) and mycolipenic acid (Panel D). Panel E- The sulfolipid 2,3,6,6'-Tetra-O-acyltrehalose 2' sulfate.

Structurally, LAM is a multi-glycosylated extension of phosphatidylinositol (PI) (Figure 1.6). The acyl chains on PI are typically palmitate and tuberculostearate, representative of the cytoplasmic membrane fatty acid content[22]. The PI residue is substituted with α-D-mannose on the 2 and 6 positions of inositol[23]. The linear regions of LAM (α-(1-6)-

linked D-mannose with α-(1-2) linked mannosyl sidechains) extend from the mannose residue on the 6 position of inositol[23]. The linear regions of arabinan are similar to

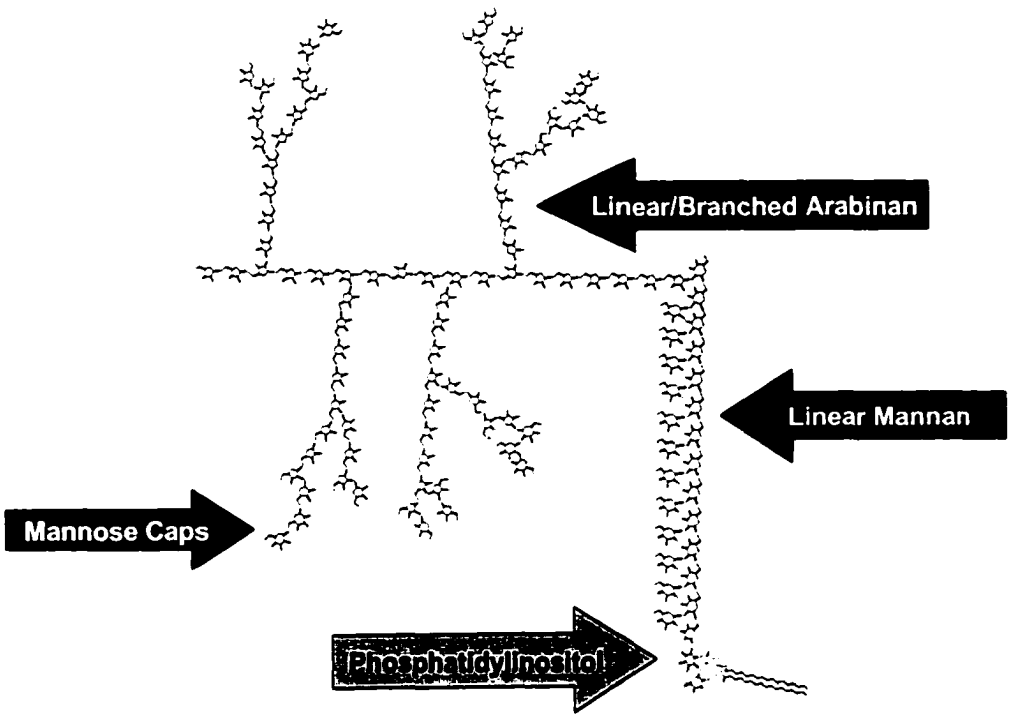

Figure 1.6- Lipoarabinomannan from *M. tuberculosis*. See text for description.

that observed in arabinogalactan, α-(1-5) linked arabinofuranose with branching introduced by 3,5-α-D-arabinofuranosyl residues[24]. There are two major arabinofurnanosyl motifs on the non-reducing termini of LAM[24]. The first is [β-D-arabinofuranosyl-(1-2)-α-D-arabinofuranosyl]$_2$-3,5-α-D-arabinofuranosyl-(1-5)-α-D-arabinofuranose, which is similar to that observed on

arabinogalactan. But LAM also has a tetraarabinosyl motif that is not found in arabinogalactan; β-D-arabinofuranosyl-(1-2)-α-D-arabinofuranosyl-(1-5)-α-D-arabinofuranosyl-(1-5)-α-D-arabinofuranose. Some of the terminal arabinosyl residues are terminated with one, two or three additional mannose residues[25].

D. Cell Wall Synthesis in the context of polyprenyl phosphate

Introduction- Peptidoglycan is the defining carbohydrate component of eubacterial cell walls. The only eubacteria that lack peptidoglycan are the *Mycoplasmatales*[26]. There are many other carbohydrate polymers found within the eubacteria in addition to peptidoglycan. For example, teichoic acid is found in Gram positive bacteria, lipopolysaccharide is found in Gram negative bacteria, and arabinogalactan (discussed above) is present in *Mycobacteria*, *Nocardia* and *Corynebacteria*. A uniting feature of cell wall polymers is that they share a common biosynthetic scheme requiring Pol-P[26-31]. Pol-P transfers mono, di- and tri- and oligosaccharides across the cytoplasmic membrane to be assembled into polymers. Interestingly, eukaryotes also contain a similar isoprenoid molecule, dolichyl phosphate, which carries sugar residues into the lumen of the endoplasmic reticulum where they can be used for glycoprotein synthesis[32].

Pol-P may also be a regulatory factor for cell wall synthesis. Experiments with cell free preparations of *Staphylococcus aureus*[33] and *Bacillus* spp.[27] have shown that Pol-P availability is rate-limiting for cell wall synthesis. It has also been suggested that the rate of formation of Lipid I in *E. coli* (Lipid I is a precursor of peptidoglycan described in the following section) may be dependent on the pool levels of Pol-P[34]. Further, cell extracts of *M. tuberculosis* (slow-growing) synthesizes Pol-P at much slower rates than *M. smegmatis* (fast-growing)[35].

The bacterial Pol-P molecule is typically *ω,diE,polyZ*-undecaprenyl phosphate[28;36], although a few exceptions have been reported in *Paracoccus denitrificans*[37] (*ω,diE,polyZ*-nonaprenyl phosphate), *M. smegmatis*[38;39] (heptaprenyl phosphate containing four saturated and three Z double bonds or four saturated and one E and two Z double bonds, and *ω,E,polyZ*-decaprenyl phosphate) and *M. tuberculosis*[40] (decaprenyl phosphate, stereochemistry undefined)(Figure 1.7). The roles of Pol-P in cell wall synthesis are discussed below.

Peptidoglycan Synthesis- As peptidoglycan is nearly ubiquitous, common biosynthetic mechanisms are expected to exist in all peptidoglycan-containing organisms. While very little research has been conducted in mycobacteria regarding

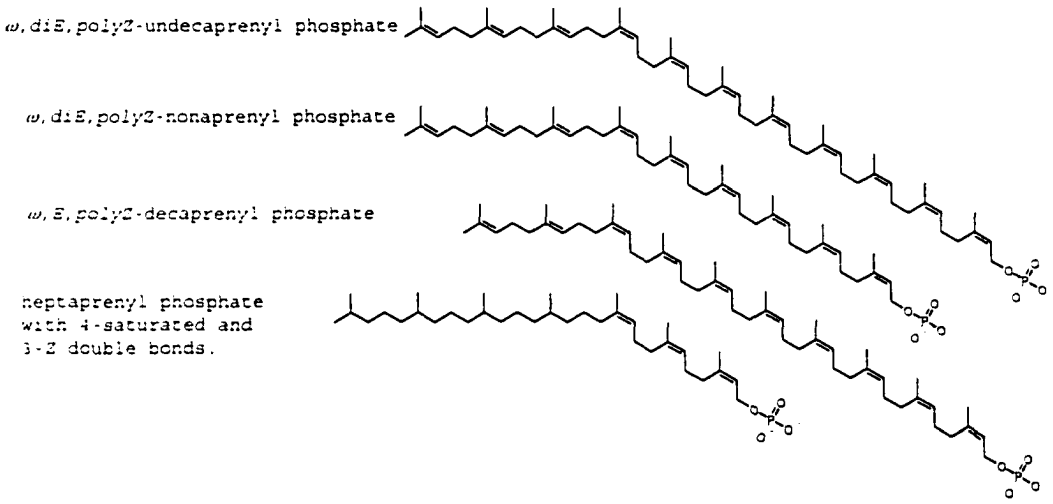

Figure 1.7- Polyprenyl phosphates from various eubacteria. *ω,diE,polyZ*-undecaprenyl phosphate is the sugar carrier molecule for most bacteria. Exceptions have been found including, *ω,diE,polyZ*-nonaprenyl phosphate (*Paracoccus denitrificans*), *ω,E,polyZ*-decaprenyl phosphate (*M. smegmatis*) and heptaprenyl phosphate with four saturated and three *Z* isoprene units. (*M. smegmatis*).

peptidoglycan synthesis, it is expected to resemble the schemes outlined for *E. coli* by van Heijenoort[34]. Peptidoglycan synthesis can be divided into two parts, 1) the synthesis of Lipid II, the peptidoglycan building block derived from Pol-P, and 2) assembly of the building blocks into mature peptidoglycan with subsequent recycling of Pol-P.

Synthesis of Lipid II[34] (Summarized in Figure 1.8)- An aminotransferase, transacetylase and a phosphomutase catalyze the synthesis of N-acetylglucosamine-1-phosphate (GlcNAc-1-P)

from fructose 6-phosphate. GlcNAc-1-P then attacks the sugar nucleotide, uridine triphosphate, displacing the pyrophosphate moiety and forming the peptidoglycan precursor uridine diphosphate (UDP)-GlcNAc. Some of the UDP-GlcNAc is

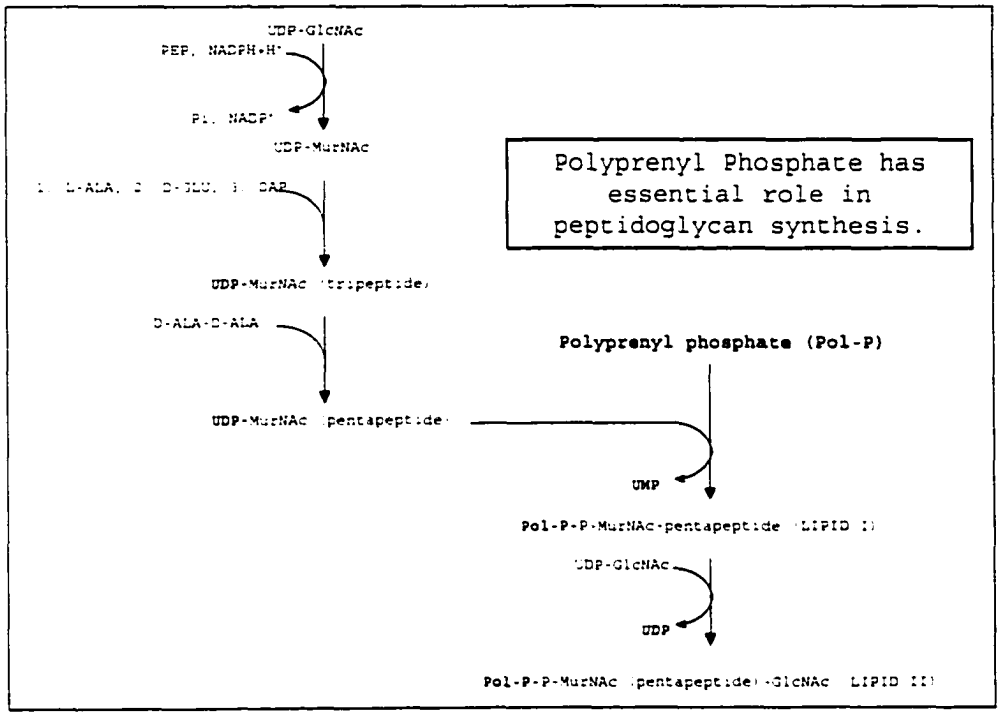

Figure 1.8- The synthesis of Lipid II in peptidoglycan synthesis. See text for description.

used directly for peptidoglycan synthesis, while some is used to form UDP-N-acetylmuramic acid (UDP-MurNAc). UDP-MurNAc is synthesized by the addition of phosphoenolpyruvate (PEP) to the C_3-hydroxyl of UDP-GlcNAc. In this reaction the phosphate on the α-carbon of PEP is displaced and the enol is reduced by NADPH. The complete UDP-MurNAc-pentapeptide is formed by the stepwise addition of L-alanine (L-ALA), D-isoglutamate (D-Glu), meso-diaminopimelic acid (DAP), and D-alanyl-D-alanine (D-ALA-D-ALA) to UDP-MurNAc. Following its formation, the

UDP-MurNAc pentapeptide is transferred to Pol-P to form Pol-P-P-MurNAc pentapeptide (Lipid I). UDP-GlcNAc is then transferred to Lipid I to form Pol-P-P-MurNAc(pentapeptide)-β-1,4-GlcNAc (Lipid II). Lipid II can then transverse the membrane (by an unknown mechanism) and participate in the transglycosylation reaction.

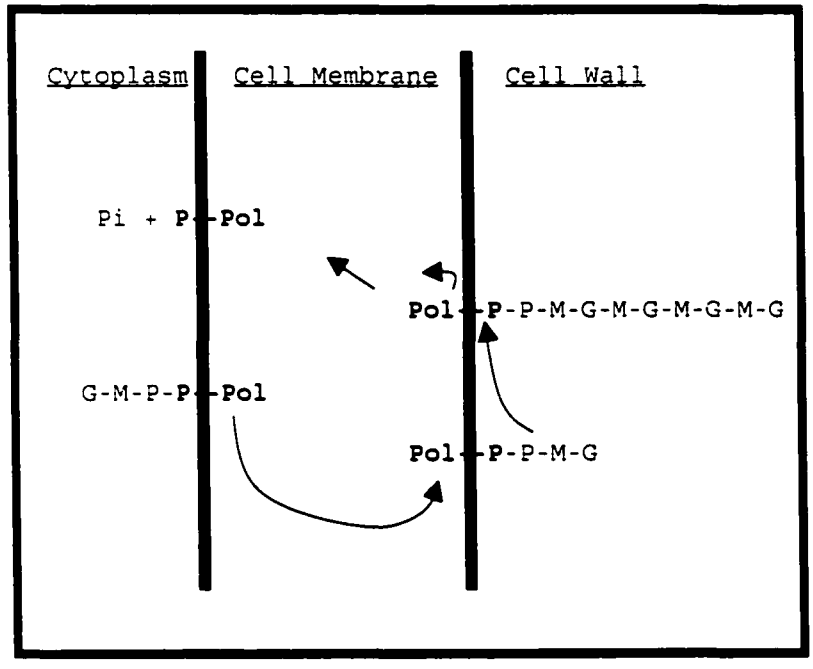

Figure 1.9- Synthesis of the glycan chain for peptidoglycan. See text for details.

Synthesis of Glycan Chain[34] (Summarized in Figure 1.9)- In the transglycosylation reaction, C_4 of GlcNAc (of Lipid II) attacks the MurNAc present in the growing glycan chain. The lipid carrier from the growing glycan chain is displaced as the new glycosidic bond is formed. However, the remaining Pol-P-P molecule of the incoming Lipid II anchors the glycan chain to the membrane. The hydrolysis of Pol-P-P to Pol-P + Pi regenerates the pool of Pol-P necessary for

continued peptidoglycan synthesis[41]. The glycan chain is integrated into the existing murein through peptide cross-links. The amino group of DAP (3rd position on the peptide) attacks the carbonyl group between the terminal D-ALA residues (4th and 5th positions of peptide) of a separate glycan chain. A new DAP-ALA peptide bond is formed while the terminal ALA is released. Mycobacteria also catalyze the formation of DAP-DAP cross-links[17], however, the mechanism by which this occurs is unknown. It is also not known precisely when or what percentage of the N-acetyl groups on muramic acid are oxidized to N-glycolyl groups. It has also been shown that free carboxyl groups of some D-GLU and DAP residues are amidated, and that a small percentage of the D-GLU residues can be substituted with glycine[42].

Arabinogalactan Synthesis- The synthesis of the linker disaccharide and the complete AG polymer occurs while attached to Pol-P (Figure 1.10)[29;30]. First, GlcNAc-1-Phosphate is transferred from UDP-GlcNAc to Pol-P by GlcNAc-1-phosphotransferase to form Pol-P-P-GlcNAc (Glycolipid I). A rhamnose residue is then transferred to glycolipid I from TDP-rhamnose to form Pol-P-P-GlcNAc-Rha (Glycolipid II). Then, glycolipid II serves as the acceptor of D-galactofuranosyl residues and D-arabinofuranosyl residues donated from the respective donors, UDP-galactofuranose[30;43] and Pol-P-arabinofuranose[38].

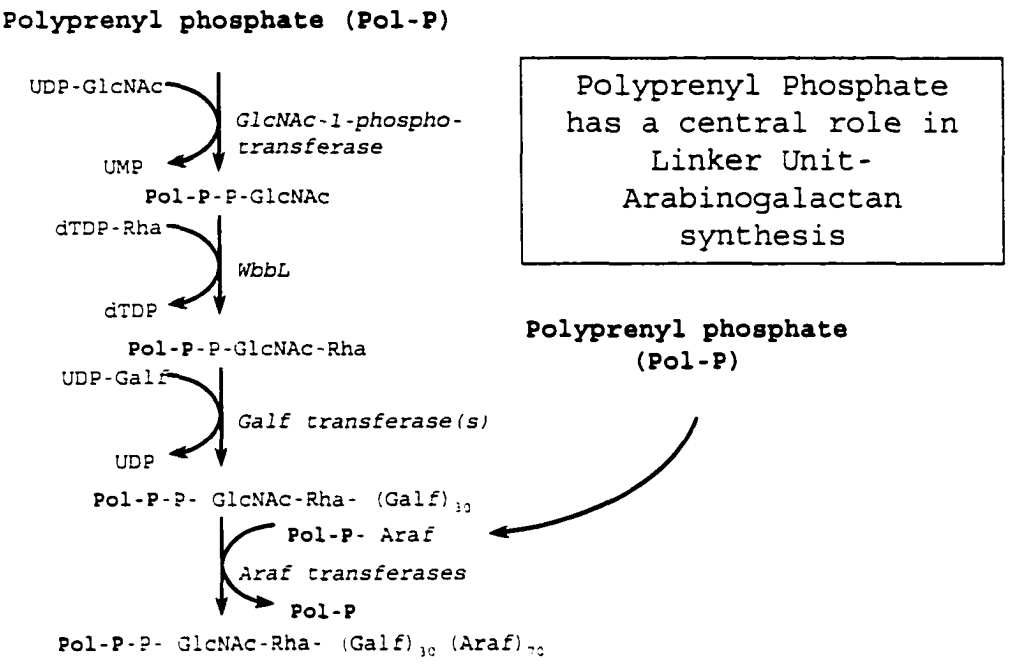

Figure 1.10- The biosynthesis of arabinogalactan and the linker disaccharide. See text for details.

Mycolic Acid Synthesis (reviewed[12;20;44])- Mycolic acid synthesis has been divided into four steps: 1) synthesis of the α-chain, the C_{24}-C_{26} straight chain saturated fatty acid, 2) synthesis of unsaturated, cyclopropanated long chain (~C_{40}-C_{60}) meromycolate, 3) further modification of the meromycolate to contain other functional groups, 4) final condensation between the α-chain and the meromycolate.

The α-chain is a product of the fatty acid synthesis pathway. Both the type I and type II fatty acid synthetase systems are present in *Mycobacteria*. The type I system is capable of

synthesizing a C_{16} fatty acid from acetate. On the other hand, the type II system is not capable of de novo synthesis, but rather is capable of elongating C_{16} fatty acids to C_{24}-C_{26}. The first committed step for meromycolate synthesis, the unsaturation of the fatty acid by a delta-5 desaturase, is followed by multiple rounds of fatty acid chain elongation with acetate. The final step of mycolic acid biosynthesis is a Claisen condensation of the meromycolate with the α-chain to yield the β-oxomycolate which is then reduced to the mature mycolate. It has been proposed that the mature mycolic acid may be formed while attached to one of three carriers, a Pol-P derivative, polyprenyl-phosphorylmannose[45], trehalose monomycolate or trehalose dimycolate[46]. Still, others suggest the existence of a yet, undefined mycolate carrier[44]. However, trehalose monomycolate was shown to be a substrate of the antigen 85 complex, which possesses mycolyl transferase activity[47].

LAM synthesis- Though not part of the mycolyl-arabinogalactan-peptidoglycan cell wall core, LAM is still a defining component of the mycobacterial cell wall. The initial steps in LAM biosynthesis involve the transfer of mannose to phosphatidylinositol to form phosphatidylinositol dimannoside (PIM_2)[48]. The mannosyl donor for these steps is guanidine diphosphate (GDP)-mannose. PIM_2 is then the acceptor for additional mannosyl residues to form linear α(1-6)-

lipomannan[45]. These steps involve the Pol-P derivative mannosyl-1-phosphoryl-decaprenol. Mature lipomannan is generated by the addition of α(1-2)mannosyl branches from GDP-mannose or DPM. The arabinose residues are donated form arabinosyl-1-phosphoryl-decaprenol[49].

E. Summary

Pol-P is a key player in mycobacterial cell wall synthesis. The synthesis of mycobacterial peptidoglycan, arabinogalactan and lipoarabinomannan require Pol-P. The essentiality of Pol-P is demonstrated by studies which have shown that *M. tuberculosis*[50] among other *Mycobacterium* spp. (Schulbach and Crick, unpublished) are sensitive to the cyclic peptide antibiotic, bacitracin, and by studies showing the sensitivity of cell free extracts of *M. smegmatis* to the lipopeptide antibiotic, amphomycin[48]. Bacitracin inhibits Pol-P-P hydrolysis by forming a complex with the diphosphate moiety[51] while amphomycin specifically binds Pol-P, preventing it from participating in glycosyl transferase reactions[52].

The Pol-P biosynthetic enzymes may be good candidates for the rational design of new chemotherapeutic agents. However, little is known about them. This dissertation summarizes my investigation of isoprenoid biosynthetic enzymes in *M. tuberculosis*. The literature review section of this dissertation will continue with Chapters 2 and 3, the

introductions to isoprenoids and isoprenyl diphosphate synthases, respectively.

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Chapter 2.

Literature Review Part II- Isoprenoid Compounds

A. Introduction

Isopentenyl diphosphate (IPP) is the precursor of all isoprenoids[1]. This branched C_5 unit is subject to polymerization and modification by the various enzymes of the isoprenoid biosynthetic pathway producing the most chemically and structurally diverse class of compounds known[2,3]. Isoprenoid compounds perform numerous essential roles in many areas of cellular metabolism.

Isoprenoid biosynthesis can be conveniently divided into three parts: 1) synthesis of the precursor, IPP, 2) chain elongation, and 3) chain modification. Since 1950, it was believed that all organisms synthesized IPP from the mevalonate pathway[4]. Recently, it has been shown that IPP can be synthesized by an alternate, mevalonate independent pathway in some organisms[4-6], including mycobacteria[7]. Once IPP is synthesized it is attached to an allylic diphosphate to generate a longer allylic diphosphate. The enzymes responsible for this chain elongation mechanism are called isoprenyl diphosphate synthases[8]. After the allylic prenyl chain has been synthesized it can undergo a variety of modifications, including but not limited to 1) a head to head condensation with a second isoprenoid chain, 2) a condensation to a non-

isoprenoid compound, 3) cyclization, 4) dephosphorylation or 5) saturation of the allylic double bonds.

B. Isoprenoid Biosynthesis Part I- Synthesis of IPP

This discussion of isoprenoid biosynthesis begins with the synthesis of the five carbon precursor, IPP. In eukaryotes, IPP is a product of the mevalonate dependent pathway[2] (Figure 2.1). The first step is the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase. HMG-CoA reductase then catalyzes the irreversible formation of mevalonate. HMG-CoA reductase is one of the most stringently regulated enzymes known and is the enzymatic target for the cholesterol lowering drug, lovastatin[3]. Mevalonate is then subject to two ATP-dependent phosphorylation events yielding 5-pyrophosphomevalonate. Finally, pyrophosphomevalonate decarboxylase catalyzes the decarboxylation of 5-pyrophosphomevalonate to IPP. This step requires ATP and probably begins with the phosphorylation of the 3-hydroxyl group, followed by a trans-elimination of both the phosphate and carboxyl moieties. IPP can be isomerized to dimethylallyl diphosphate (DMAPP), the C_5 allylic diphosphate used in isoprenoid synthesis.

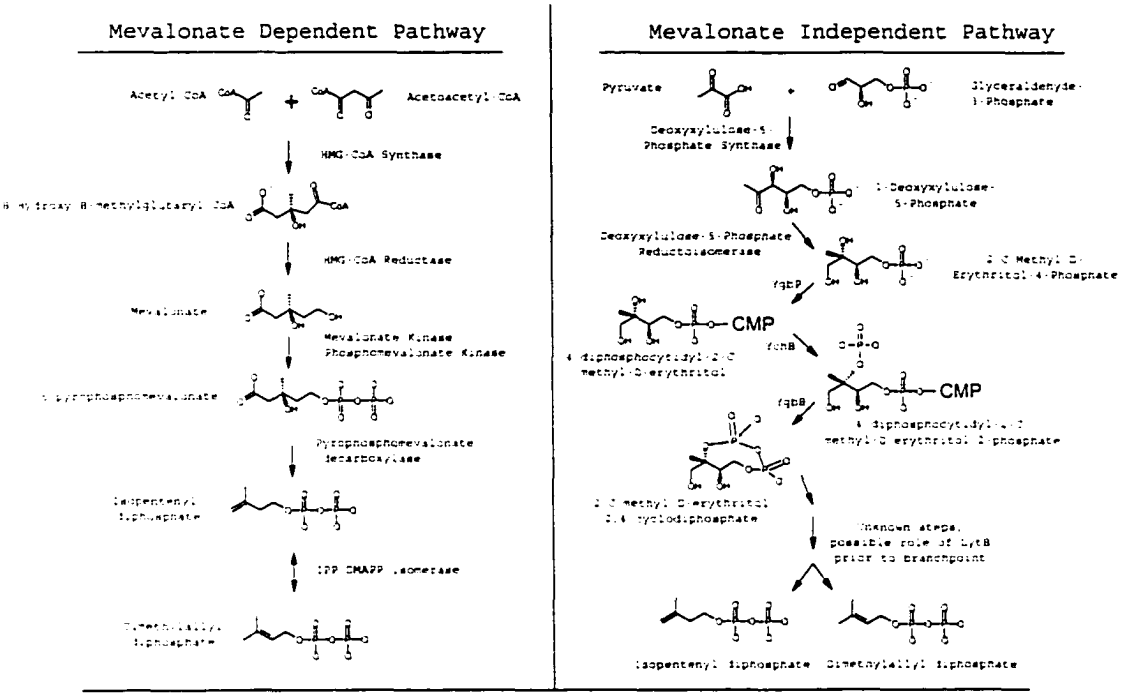


Figure 2.1 *The initial steps of Isoprenoid Biosynthesis.* The Mevalonate Dependent Pathway (left) and the Mevalonate Independent Pathway (right)are both capable of synthesizing the universal precuror of isoprenoids, isopentenyl diphosphate.

Algae, some bacteria and the chloroplasts of higher plants, have been shown to use an alternate mevalonate independent pathway for the synthesis of IPP (Figure 2.1)[6;7;10]. The first step involves the condensation of pyruvate with glyceraldehyde 3-phosphate (or glyceraldehyde) to form 1-deoxy-D-xylulose 5-phosphate (or 1-deoxy-D-xylulose). The enzyme which catalyzes this reaction, 1-deoxy-D-xylulose 5-phosphate synthase, has been cloned from various sources[11-14] including *M. tuberculosis* (Bailey and Crick, personal communication). 1-Deoxy-D-xylulose 5-phosphate reductoisomerase then catalyzes the formation of 2-C-methyl-D-erythritol 4-phosphate[4]. Three gene products have been identified that catalyze reactions downstream of the 1-

deoxy-D-xylulose 5-phosphate reductoisomerase, YgbP protein catalyzes the formation of 4-diphosphocytidyl-2-C-methylerythritol[15], and YchB protein phosphorylates the 2-hydroxy group of 4-diphosphocytidyl-2C-methylerythritol[16] and YgbB protein converts 4-diphosphocytidyl-2C-methylerythritol 2-phosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate[17]. In contrast to the mevalonate pathway, evidence shows the mevalonate independent pathway may branch and separately produce DMAPP and IPP[18;19].

C. Isoprenoid Biosynthesis Part II- Chain Elongation

DMAPP is the first allylic substrate for chain elongation. Chain elongation occurs via syn addition, in which the π-electrons of IPP attach to carbon-1 of DMAPP[2](Figure 2.2) to generated a new single bond. One of the protons (H_R or H_S) on

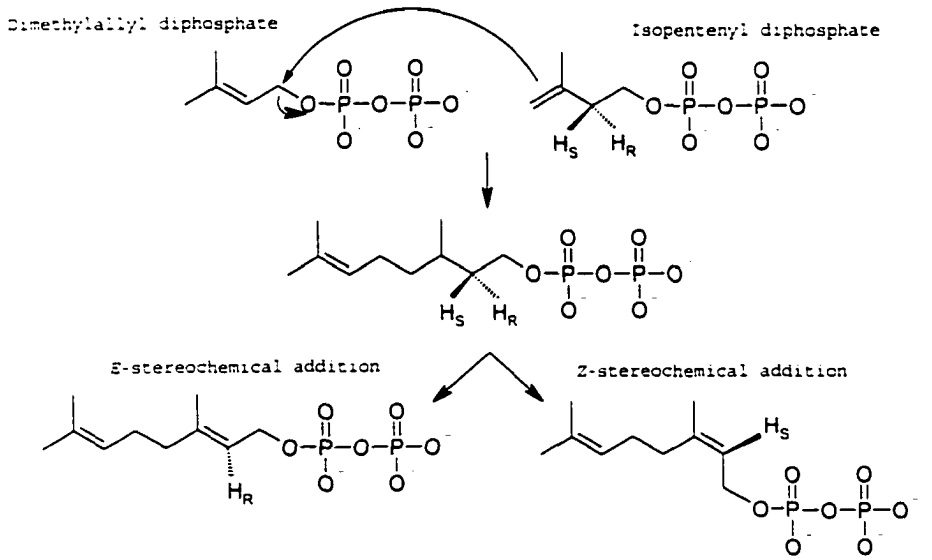


Figure 2.2 *The Mechanism of Chain Elongation.* The π-electrons of isopentenyl diphosphate attach to C_1 of dimethylallyl diphosphate. The elimination of either the H_S or the H_R proton completes an *E*- or a *Z*-stereochemical addition, respectively.

C_3 of IPP is eliminated, forming a new double bond with *E*-or Z-stereochemistry, respectively[3;20]. In discussions of a prenyl diphosphate chain, each unit has its stereochemistry designated starting from the ω end of the molecule (Figure 2.3).

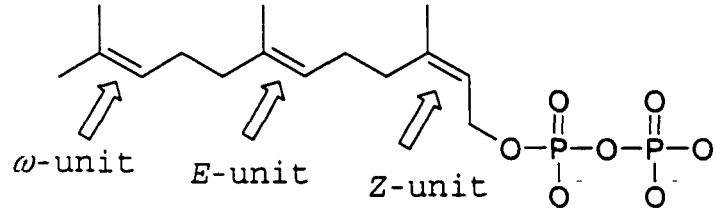


Figure 2.3 *Isoprene stereochemistry.* The isoprene unit furthest from the diphosphate moiety (left) has no stereochemistry and is called an ω isoprene unit. An isoprene unit with *E*-stereochemistry has the methyl and hydrogen substituents on opposite sides of the double bond (center). An isoprene unit designated to have *Z*-stereochemistry has the methyl and hydrogen substituents on the same side of the double bond (right).

Isoprenyl diphosphate synthases are specific for the type of double bond formed (*E* or *Z*)[1]. For example, when DMAPP and IPP condense, they may form either ω,*E*-geranyl diphosphate (GPP) (by an *E*-isoprenyl diphosphate synthase) or its stereoisomer ω,*Z*-neryl diphosphate (NPP) (by a *Z*-isoprenyl diphosphate synthase). Longer prenyl chains (farnesyl diphosphate, FPP (C_{15}), geranylgeranyl diphosphate, GGPP (C_{20}), geranylfarnesyl diphosphate (C_{25}), hexaprenyl diphosphate (C_{30}) etc.) are synthesized by further additions of IPP to the allylic diphosphate (Figure 2.4).

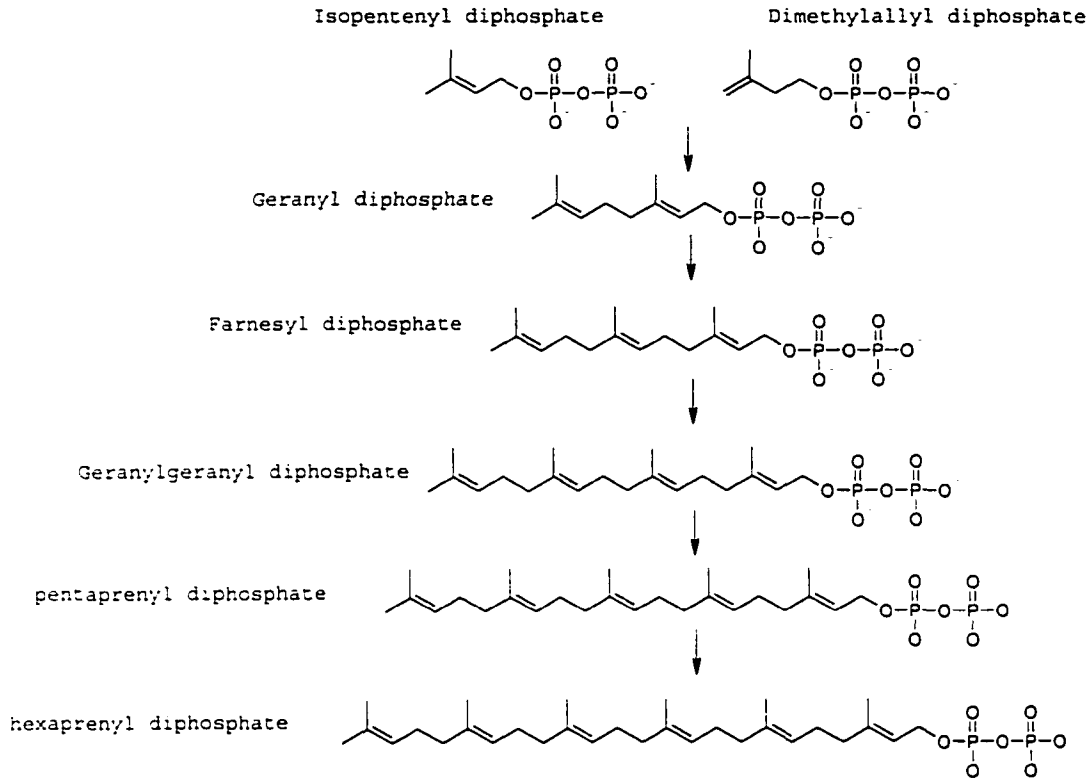


Figure 2.4 Chain elongation of allylic diphosphates with isopentenyl diphosphate (IPP) to form longer allylic diphosphates. In order to simplify the figure, isoprene units are only added in the *E*-stereoconfiguration.

The chain elongation pathway can utilize both *E*- and *Z*-isoprenyl diphosphate synthases to create an isoprenoid chain with mixed stereochemistry. *ω,diE,polyZ*-Undecaprenyl phosphate, an isoprenoid with a central role is prokaryotic cell wall biosynthesis (discussed in Chapter 1) is an example of an isoprenoid with mixed (*E* and *Z*) stereochemistry[21]. *ω,diE,polyZ*-Undecaprenyl diphosphate is generated by two chain

elongation enzymes, 1) an *ω,E,E*-farnesyl diphosphate synthase which synthesizes *ω,E,E*-farnesyl diphosphate from DMAPP and two molecules of IPP and 2) a *Z*-undecaprenyl diphosphate synthase which uses *ω,E,E*-farnesyl diphosphate as the allylic substrate

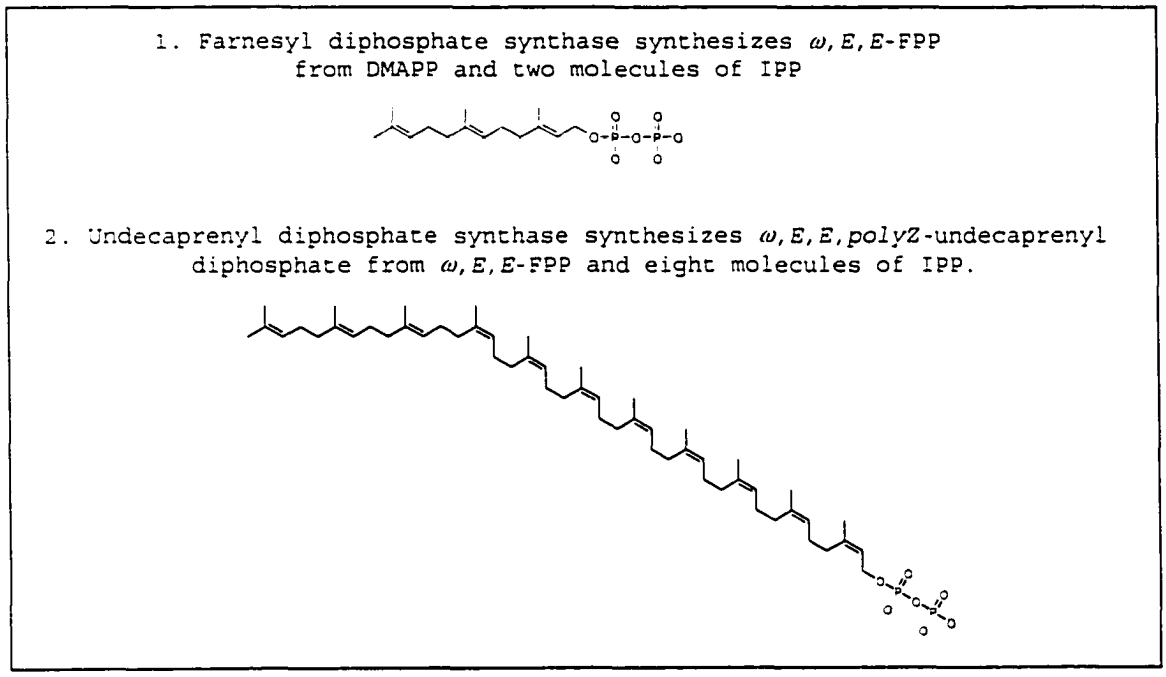


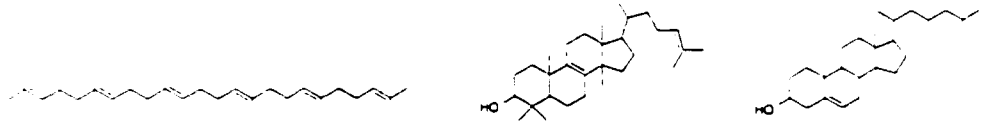
Figure 2.5- E- and Z- isoprenyl diphosphate synthase work together to create isoprenoid molecules with mixed stereochemistry.

and adds eight molecules of IPP with *Z*-stereochemistry (Figure 2.5). *ω,E,E,polyZ*-Undecaprenyl diphosphate is the precursor of *ω,E,E,polyZ*-undecaprenyl phosphate, which functions as the lipid carrier molecule for the synthesis of cell wall macromolecules[22].

D. Isoprenoid Biosynthesis Part III- Chain Modification

Once the prenyl chain has reached a physiologically relevant length, it may undergo certain modifications to yield the final isoprenoid molecule. Among the possible modifications are dephosphorylation (mentioned above), desaturation, condensation, or cyclization reactions.

Cholesterol (Figure 2.6) is a major component of eukaryotic membranes and is the precursor of steroid hormones and bile acids. Cholesterol synthesis (Figure 2.5) requires the head to



Squalene Lanosterol Cholesterol

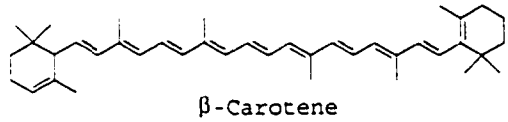
Figure 2.6 *Key intermediates in cholesterol synthesis .*

head condensation of two farnesyl diphosphate molecules to form squalene. Squalene is subsequently cyclized to lanosterol, which is then reduced and demethylated to cholesterol. New evidence suggests that *M. tuberculosis* synthesizes cholesterol[23]. In addition, *M. tuberculosis* has demonstrated the ability to uptake, modify, and accumulate cholesterol from liquid growth media, and to form a zone of clearance around a colony when plated on solid media containing cholesterol[24]. Combined with the observation that the presence of cholesterol

in macrophages is required for the tubercle bacillus to enter the macrophage[25], one is faced with the question: Could cholesterol biosynthesis be a virulence factor for *M. tuberculosis*?

In light of the above question, only two prokaryotes have been identified that synthesize cholesterol, *Methylococcus capsulatus* and *Nannocystis exedens*[26]. However, some prokaryotes do contain related, pentacyclic isoprenoid molecules called hopanoids. It has been postulated that hopanoids, like cholesterol, play a role in membrane stabilization[27;28]. It is unknown whether hopanoids exist in mycobacteria.

Carotenoids (Figure 2.7) are a family of about three hundred isoprenoids that are capable of quenching oxygen radicals[29]. It

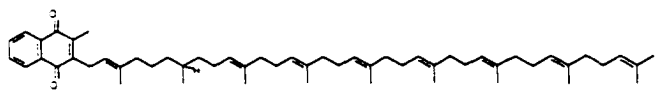


β-Carotene

Figure 2.7 The structure of β-Carotene, a representative carotenoid.

has also been suggested that carotenoids substituted with polar groups are incorporated into membranes where they have a mechanical role similar to that described for cholesterol and hopanoids[26]. The spectrum of colors observed throughout the different carotenoid species is dependent upon the number and location of conjugated double bonds. Carotenoid synthesis

begins with a head to head condensation of two geranylgeranyl diphosphate molecules to form phytoene, which then is subjected to modifications such as cyclization and desaturation. Carotenoids have been isolated from many different species of mycobacteria, including *M. tuberculosis*. Some species of mycobacteria only synthesize carotenoids after light induction, whereas other mycobacteria synthesize them constituitively. A carotenoid biosynthetic gene cluster has been cloned from *Mycobacterium aurum*[30]. This cluster contains eight strictly regulated genes. A similar cluster has not been identified in *M. tuberculosis*. Of those mycobacteria capable of causing disease, carotenoids have been postulated to protect the bacilli from potential oxidative damage released from the host cell lysosomes[31].



Menaquinone - 9

Figure 2.8 The structure of the major menaquinone component from *Mycobacterium phlei*. There are nine isoprene unit in the prenyl sidechain. The second isoprene unit (from the naphthoate head group) is saturated.

Menaquinone (Figure 2.8) is a lipid electron carrier essential for aerobic respiration and its biosynthesis was reviewed by Minnikin[32]. It is synthesized by the condensation of a naphthoate head group with prenyl diphosphate. Depending on the species, the prenyl tail may have between six and ten isoprene units[29]. Mycobacterial menaquinone contains nine

isoprene units with the second isoprene unit from the head group being saturated (Figure 2.8).

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Chapter 3.

Literature Review Part III- Isoprenyl Diphosphate Synthases

A. Introduction

Isoprenyl diphosphate synthases catalyze the chain elongation of IPP to allylic prenyl diphosphates of various chain lengths (discussed in Chapter 2). These enzymes are specific for the type of stereochemistry (*E* or *Z*) formed in the new allylic double bond. Amino acid sequence information is available for about fifty *E*-isoprenyl diphosphate synthases[1,2] and only a few *Z*-isoprenyl diphosphate synthases[3,4]. Amino acid alignments have revealed that there is absolutely no amino acid sequence homology between *E*- and *Z*-isoprenyl diphosphate synthases[5]. However, there is a great deal of homology among the members of the *E*-isoprenyl diphosphate synthase family[6]. Evolutionary implications have been derived from the amino acid sequences of *E*-isoprenyl diphosphate synthases[6].

B. *E*-Isoprenyl Diphosphate Synthases

Amino acid sequence alignments reveal at least five conserved regions among *E*-isoprenyl diphosphate synthases (Figure 3.1)[1,2]. The major conserved feature that defines the *E*-isoprenyl diphosphate synthase family is presence of two aspartate rich motifs, $DD(XX)_{1-2}D$. The first aspartate rich motif (FARM, region II) and the second aspartate rich motif (SARM, region V) coordinate Mg^{2+} ions in the active site that

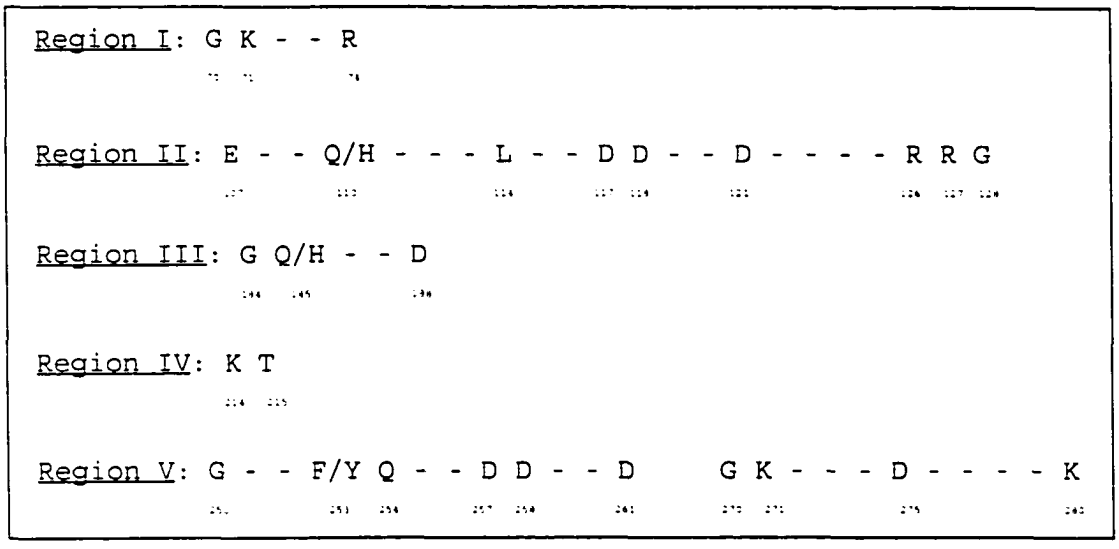

Figure 3.1- The conserved regions of *E*-isoprenyl diphosphate synthases. The amino acids are numbered from their positions in the avian FPP synthase.

function to suppress the negative charges of the diphosphate moieties of the substrates[7;8]. Two arginine residues following the FARM motif (in region II) are also essential[2;8]. It has been postulated that they stabilize the diphosphate leaving group during the formation of the carbocation on C-1 of the allylic diphosphate.

The crystal structure of avian *E*-farnesyl diphosphate synthase has been resolved[9]. The protein was shown to possess 10 α-helices and a large barrel like cavity. This structural motif has been named the "isoprenoid synthase fold" and is also common to the isoprenoid cyclase enzymes, whose crystal structures have also been resolved[10-12]. The conserved aspartate residues in the FARM and the SARM are present on

opposite walls of the central cavity. The other conserved regions identified from amino acid sequence alignments are also located near the cavity. From the crystal studies it is believed that each addition of IPP to the allylic diphosphate substrate pushes the allylic diphosphate deeper into the barrel-like cavity. Once the barrel-like cavity is unable to bind the growing allylic diphosphate, the product is released. Amino acids in and around the FARM motif sterically regulate the depth of the cavity, and therefore the chain length of the product[8].

Wang and Ohnuma have suggested that all *E*-isoprenyl diphosphate synthases evolved from a common ancestor and the gross topological features of the original enzyme have been conserved[5]. In Wang and Ohnuma's model, the bifunctional Archaeal FPP/GGPP synthase is the closest relative of the ancestral enzyme, and four mutational events occur, producing new enzyme classes. The five different types of *E*-isoprenyl diphosphate synthases are discussed below and their relationships are outlined in Figure 3.2.

GGPP synthase type I- The GGPP synthase type I binds the allylic substrate, DMAPP, adds 2-3 molecules of IPP and releases both FPP and GGPP[6;13]. It is thought to represent the ancestral *E*-isoprenyl diphosphate synthase. The main features among the members of the GGPP synthase type I family are 1)

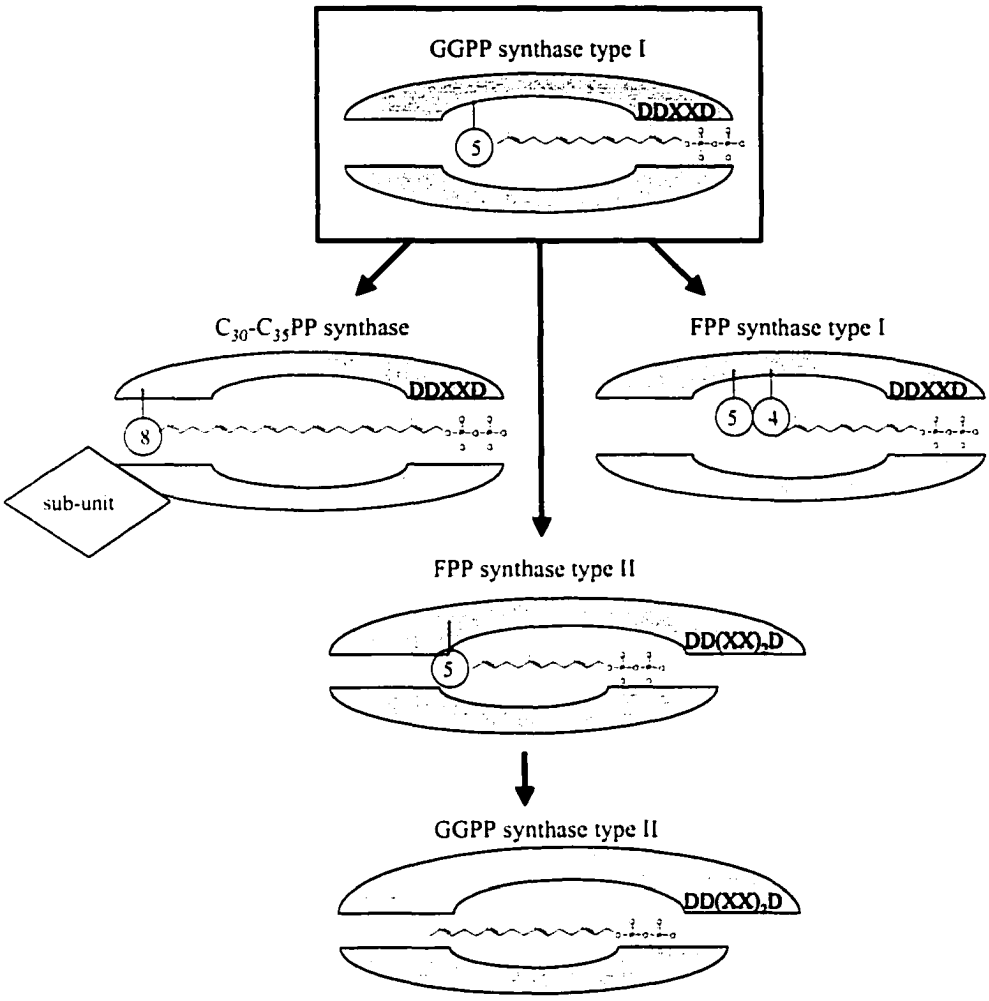

Figure 3.2- *The five types of E-isoprenyl diphosphate synthases and their evolutionary lineage as described by Wang and Ohnuma.* From the GGPP synthase type I there are three major mutational events, including 1) the addition of an aromatic amino acid to produce FPP synthase type I, 2) loss of an aromatic amino acid to produce a C_{30}-C_{35}-PP synthase, and 3) a two amino acid insertion in the FARM to produce the FPP synthase type II. The FPP synthase type II lost the aromatic amino acid in position -5 to produce the GGPP synthase type II. The C_{30}-C_{35}-PP synthase is a heterodimer, the small subunit does not have any conserved features of *E*-isoprenyl diphosphate synthases.

the FARM, DDXXD and 2) presence of an aromatic amino acid in position -5 before the FARM. The aromatic amino acid shapes the barrel-like cavity and regulates the product chain length. The GGPP synthase type I from *Sulfolobus acidocaldarius* was subjected to a random mutagenesis study[14]. Results showed that substitution of phenylalanine-77 (the aromatic amino acid -5 to the FARM) with a serine or leucine residue deepens the barrel-like cavity and lengthens the product.

C_{30}-C_{35} Isoprenyl diphosphate synthases- The *E*-isoprenyl diphosphate synthases catalyzing the formation of C_{30}-C_{35} products from C_{15}-C_{20} allylic primers are heterodimers[15;16]. The large subunit contains the amino acid sequence features of the *E*-isoprenyl diphosphate synthase family. It presumably evolved from the GGPP synthase type I. However, it lacks the bulky aromatic amino acid at position -5 before the FARM, and instead contains an amino acid with a small side chain (a evolutionary step reminiscent of the mutagenesis experiment conducted on the GGPP synthase type I). In position -8 before the FARM, there is a an isoleucine residue which is thought to reside in the barrel-like cavity in much the same way as the aromatic amino acid does for the GGPP synthase type I. A site-directed mutagenesis experiment with the heptaprenyl (C_{35}) diphosphate synthase from *Bacillus stearothermophilus* [17] demonstrated that the replacement of the isoleucine-76 with glycine increased the length of the product. In the same

report, the addition of an aromatic amino acid to the -5 position before the FARM decreased the product chain length from C_{35} to C_{20}. The small subunit of the medium chain isoprenyl diphosphate synthases do not contain the sequence features of the *E*-isoprenyl diphosphate synthase family. However, the small subunit is essential for catalytic activity[15;16]. In fact it was recently shown that the small subunit is involved in the binding of allylic substrate as well as determining the chain length of the reaction product[18;19].

FPP synthase type I- The FPP synthases type I (eukaryotic FPP synthases) also evolved from the GGPP synthase type I. Like the GGPP synthase type I, members of the FPP synthase type I contain the FARM, DDXXD. In contrast to the GGPP synthase type I which contain only one aromatic amino acid (in position -5 to the FARM), the FPP synthases type I contain two aromatic amino acids at the positions -4 and -5 to the FARM. A mutant *Sulfolobus acidocaldarius* GGPP synthase type I was created in which an additional aromatic amino acid was placed -4 to the FARM[20]. This change shortened the barrel-like cavity and reduced product length to C_{15}, reflecting the suggested evolutionary course of the FPP synthases type I.

FPP synthase type II- The FPP synthases type II are found in prokaryotic organisms and are thought to have evolved from the

GGPP synthase type I. Reminiscent of the GGPP synthase type I, the FPP synthase type II contains an aromatic amino acid at position -5 before the FARM. The defining evolutionary feature of the FPP synthase type II is the two amino acid insertion inside the FARM motif, DDX$\underline{XX}$XD[6]. Some other unknown changes also occurred that shortened the barrel-like cavity, shortening the product chain length from C_{20} to C_{15}[20].

GGPP synthase type II- These enzymes presumably evolved from the FPP synthase type II. The depth of the barrel-like cavity was increased following the loss of the aromatic amino acid at position -5 before the FARM. Like its progenitor, the GGPP synthase type II contains the two amino acid insertion in the FARM, DDX$\underline{XX}$XD[6].

C. *Z*-Isoprenyl Diphosphate Synthases

Z-isoprenyl diphosphate synthases bind ω,E,E-FPP and ω,E,E,E-GGPP as substrates and release long chain products with mixed (*E* and *Z*) stereochemistry[21;22]. These enzymes traditionally have been synonymous with long chain isoprenyl diphosphate synthases[2]. In comparison to *E*-isoprenyl diphosphate synthases, relatively little is known about *Z*-isoprenyl diphosphate synthases. In fact, the first amino acid sequence for a *Z*-isoprenyl diphosphate synthase was reported in 1998, when Shimizu *et al.* cloned and characterized undecaprenyl diphosphate synthase (UPS) from *Micrococcus luteus*[3]. Since

then, UPS homologs have been cloned from *E. coli*, *Streptococcus pneumoniae*, *Haemophilus influenzae*[4;23]. In addition, the dehydrodolichol diphosphate synthase from *Saccharomyces cerevisiae* and *Arabidopsis thaliana* have now been cloned[24;25]. The conserved regions amongst these proteins do not resemble any motifs in the *E*-isoprenyl diphosphate synthases, confirming the suspicion that the two groups are not related[3;4]. Earlier studies of the partially pure *Lactobacillus plantarum* undecaprenyl diphosphate synthase revealed that a divalent cation is required for catalytic activity, perhaps suggesting that a similar mechanism is required to quench the negative charges on the diphosphate moieties in the binding site[26]. The crystallization and preliminary X-ray crystallography has now been reported for the *Micrococcus luteus* UPS, but the quaternary structure has not been published[5]. It is not known which of the conserved amino acid residues in *Z*-isoprenyl diphosphate synthase alignments are required for catalysis.

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Chapter 4.

General Methods

Many procedures were essential to the investigation of the isoprenoid biosynthetic pathway in *M. tuberculosis*. Some were unique for a particular set of experiments while others were used numerous times. The unique procedures will remain embedded in the chapters to which they belong. However, the general experimental methods were extracted from Chapters 5-8 and will be described only once, as follows:

A. Materials

[^{14}C] Isopentenyl diphosphate ([^{14}C] IPP, 55-60 mCi/mmol) was purchased from Amersham Life Science Inc. (Arlington Heights, IL). Potato acid phosphatase (grade 2) was purchased from Boehringer Mannheim (Indianapolis, IN). Kanamycin, farnesol (mixed stereoisomers), *omega, E, E*-farnesol, *omega, E*-geraniol, *omega, E, E, E*-geranylgeraniol were purchased from Sigma (St. Louis, MO). *omega, E, E*-farnesyl diphosphate and *omega, E*-geranyl diphosphate were synthesized as described by Davisson *et al.*[1]. 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 (Gibbstown, NJ) and Baker Si-Reverse Phase C18 TLC plates were from J.T. Baker (Phillipsburg, NJ). Restriction enzymes *Nde*I and *Hind*III, LB agar and LB broth, DNTPs, T4 DNA ligase were purchased from Gibco BRL, Life Technologies (Rockville, MD). QIAprep Spin Miniprep Kit was purchased from Qiagen Inc. (Santa Clarita, CA). *Escherichia coli* competent cells (XL-1 Blue) were purchased from Stratagene (La Jolla, CA). Vent Polymerase was purchased from New England Biolabs, Inc. (Beverly, MA).

B. Sub-cellular fractionation

M. smegmatis (wildtype or recombinant) or *M. tuberculosis* H37Rv (previously irradiated) cells were disrupted by probe sonication in a buffer containing 50 mM MOPS (morpholinopropanesulfonic acid) (pH 7.9), 0.25 M sucrose, 10 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. The resulting suspension was centrifuged at 15,000 X g for 15 min. The pellet was discarded and the supernatant was ultracentrifuged at 200,000 X g for 1 h in a Beckman Ti70.1 rotor. The supernatant (cytosol) was divided into 1-ml aliquots and frozen at -70°C until used. The pellets (membranes) were resuspended in the previously described buffer and stored as aliquots at -70°C until used. The protein concentrations of the fractions were estimated using a bicinchonchinic acid protein assay kit (Pierce).

C. *In vitro* Isoprenyl Diphosphate Synthase Assays

Isoprenyl 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, 0.3% Triton X-100, 100 μM allylic diphosphate, 30 μM [^{14}C]IPP and 75 μg protein (cytosolic or membrane) 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. Radiolabelled products were extracted with butanol saturated with water and an aliquot was taken for liquid scintillation spectrometry. Reactions were assayed under conditions where they were linear for both time and protein concentration. The radiolabelled products were characterized by TLC after treatment with potato acid phosphatase.

D. Enzymatic Treatment of Reaction Products

Butanol was removed under a stream of nitrogen. The radiolabelled products were dissolved in 5 ml of buffer containing 100 mM sodium acetate (pH 4.8), 0.1% Triton X-100 and 60% methanol[2]. After a brief bath sonication, 20 units of potato acid phosphatase were added and the mixture was incubated at 25°C overnight. Dephosphorylated products were extracted 3 times with 1 ml of n-hexane. The pooled extracts were washed with 1 ml of water and the extracts were evaporated under nitrogen. The samples were dissolved in 200μl chloroform/methanol (2:1, v/v)

and an aliquot was taken for liquid scintillation spectrometry. A second aliquot was spotted on TLC plates for analysis.

E. Analysis of Radiolabelled Product Chain Length

Analysis of the chain length of the dephosphorylated products was accomplished by spotting the radioactive compounds on reverse phase C_{18} TLC plates. The plates were then developed in methanol/acetone (8:2, v/v). Radioactive spots were located with a Bioscan System 200 Imaging Scanner (Bioscan Inc., Washington DC) and by autoradiography. Standard polyprenols were located with an anisaldehyde spray reagent[3].

F. Analysis of Radiolabelled Product Stereochemistry

The radioactive spots derived from enzymatically labeled, dephosphorylated products and identified as farnesol or geranylgeraniol were scraped from the reverse phase TLC plates and extracted from the gel with two 5 ml portions of chloroform/methanol (2:1, v/v). The extracts were pooled and dried under nitrogen. The radiolabelled farnesol was dissolved in chloroform/methanol (2:1, v/v) containing authentic, non-radioactive *ω,E,E*-farnesol or mixed stereoisomers (*ω,E,E* and *ω,E,Z*) of farnesol. The radiolabelled geranylgeraniol was dissolved in chloroform/methanol (2:1, v/v) containing authentic, non-radioactive *ω,E,E,E*-geranylgeraniol or *ω,E,E,Z*-geranylgeraniol. The stereochemistry of the radiolabelled products was determined by spotting on silica gel 60 TLC plates

that were 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[3].

G. Amino Acid Sequence Analysis

Protein sequences were from the National Institute for Biotechnology Information (NCBI), which can be accessed on the world wide web. BLAST searches were also performed at NCBI. Amino acid sequence alignments were performed with the Multalin interface (world wide web) and edited with Genedoc. CLUSTAL analysis was performed on the world wide web and the data was viewed and edited in TreeView version 1.6.1.

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Chapter 5.

Detection of isoprenyl diphosphate synthase activity and putative isoprenyl diphosphate synthases in *M. tuberculosis*.

A. Introduction

Takayama *et al.* isolated decaprenyl phosphate from *M. tuberculosis*, however its stereochemistry was not determined[1]. As described in Chapter 1, polyprenyl phosphate (Pol-P), or more specifically decaprenyl phosphate, is essential for mycobacterial cell wall synthesis. The enzymes involved in Pol-P biosynthesis may be good targets for the rational design of anti-mycobacterial agents. This chapter has been divided into two distinct sections: B) an examination of the isoprenyl diphosphate synthase activities present in *M. tuberculosis* cell free extracts, and C) an examination of the *M. tuberculosis* H37Rv genome sequence for open reading frames homologous to known isoprenyl diphosphate synthases.

B. Isoprenyl diphosphate synthase activity in the cell free extracts of *M. tuberculosis*

To initiate the investigation of Pol-P synthesis, isoprenyl diphosphate synthase activities were assayed in cell free extracts of *M. tuberculosis*. This data has been presented in the published manuscript entitled "Polyprenyl Phosphate

Biosynthesis in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*" (Journal of Bacteriology, 2000 Oct;182:5771-8)[2].

The cytosolic and membrane fractions prepared from *M. tuberculosis* contain enzymatic activities that incorporate radioactivity from [^{14}C]IPP into longer allylic diphosphate products using DMAPP, *ω,E*-GPP, *ω,E,E*-FPP and *ω,E,E,E*-GGPP as reaction primers. The assay outline is presented in Figure 5.1.

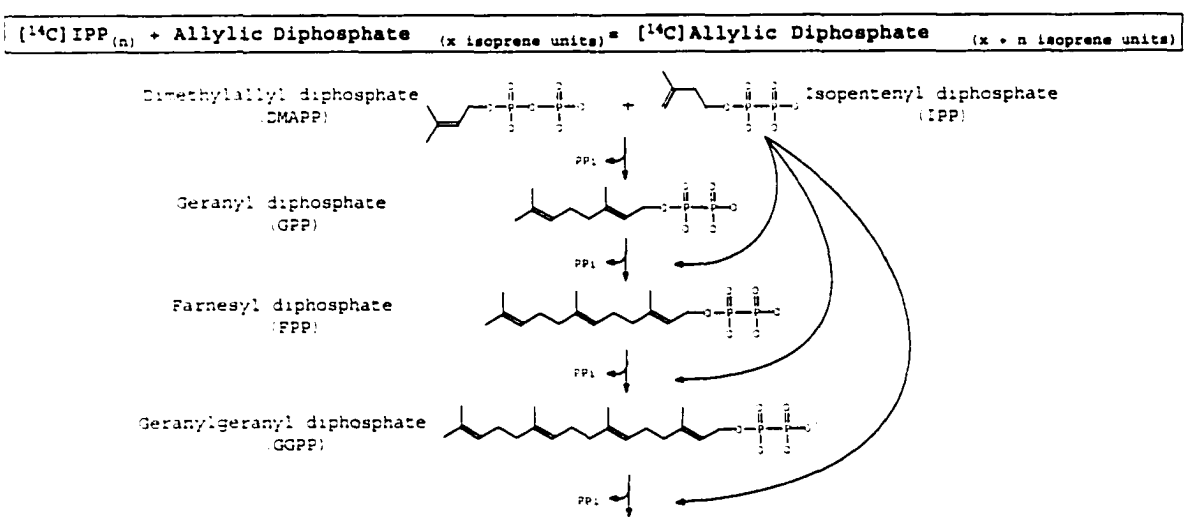

Figure 5.1- Incorporation of [^{14}C]IPP into allylic diphosphates. Assays are primed with one allylic primer (DMAPP, GPP, FPP or GGPP). Membrane or cytosolic protein with isoprenyl diphosphate synthase activity will extend the allylic primer with one or more molecules of IPP. Butanol saturated with water is used to extract the radiolabelled products. The IPP remains in the aqueous phase.

The assays were stopped with the addition of water saturated with NaCl. The radiolabelled products were extracted from the assay mixture with butanol saturated with water. An aliquot was removed for scintillation spectrometry. As shown in Table 5.1, [^{14}C] radiolabelled allylic diphosphates were formed when cytosol and membrane protein was primed with DMAPP, *ω,E*-GPP, *ω,E,E*-FPP and *ω,E,E,E*-GGPP. When no allylic primer was added to the reaction mixtures containing cytosol or membranes, no allylic diphosphates were formed.

Table 5.1- Incorporation of [^{14}C]IPP into allylic diphosphates catalyzed by cytosol or membrane fractions prepared from *M. tuberculosis* in the presence of various allylic diphosphate primers.

Allylic Primer	*M. tuberculosis*	
	Cytosol	Membranes
	(pmol/mg/min)	(pmol/mg/min)
None	0	0
DMAPP	4.0	0.6
ω,E-GPP	25.4	32.2
ω,E,E-FPP	5.8	5.7
ω,E,E,E-GGPP	0.4	1.3

Characterization of chain length of enzymatically labeled products synthesized by mycobacterial cytosol- In order to determine the chain length of the radiolabelled products synthesized by the enzymatic reactions they were dephosphorylated and analyzed by reverse-phase TLC. Although

the rates of the reactions were low, the incorporation of IPP into product was linear for up to 12 h. Analysis of the products showed that the ratios of isoprenyl diphosphates produced in each assay remained the same at all time points up to and including 12 h (data not shown). These properties allowed the generation of sufficient product for subsequent chain length and stereochemical analysis.

Figure 5.2 shows TLC analysis of [^{14}C] labeled prenols generated by dephosphorylation of isoprenyl diphosphates synthesized by *M. tuberculosis* cytosol incubated in the presence of [^{14}C] IPP and GPP. The major product was identified as geranylgeraniol (GGPP prior to dephosphorylation) with significantly smaller amounts of FPP being produced. In other experiments, no detectable GPP was produced when DMAPP was used as the primer and no radiolabelled FPP was produced when cold *ω,E,E*-FPP was used as the reaction primer (data not shown).

Characterization of chain length of enzymatically labeled products synthesized by mycobacterial membranes- The low rate of synthesis of prenyl diphosphates by *M. tuberculosis* membranes from [^{14}C] IPP and DMAPP resulted in very small amounts of product for analysis, thus preventing unequivocal

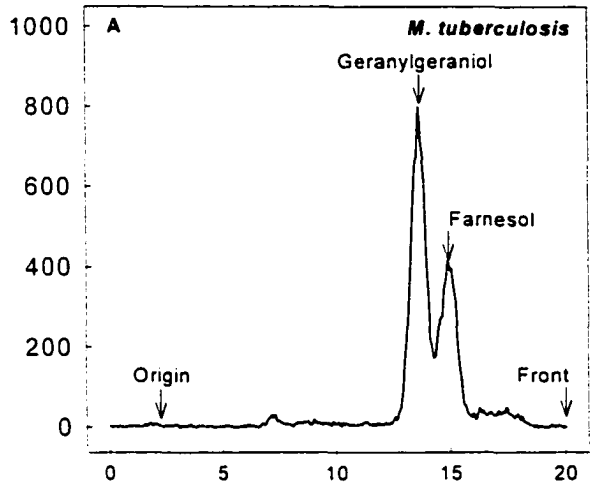

Figure 5.2- TLC analysis of products synthesized by *M. tuberculosis* cytosol in the presence of [^{14}C]IPP and *ω,E*-GPP. The radiolabelled prenyl diphosphates were dephosphorylated with potato acid phosphatase. Dephosphorylated products were analyzed on $LKC_{18}F$ TLC plates developed in methanol/acetone (8:2 v/v). Radioactive spots were located with a Bioscan System 200 Imaging Scanner (Bioscan Inc.) and standard polyprenols were located with an anisaldehyde spray reagent.

identification (Figure 5.3, Panel A). However, *M. tuberculosis* membranes incubated with *ω,E*-GPP as the reaction primer resulted in FPP and decaprenyl diphosphate being the major products, but products that correspond to prenyl diphosphates having eight and nine isoprene units were also formed (Figure 5.3, Panel B). Only geranylgeranyl diphosphate, octa-, nona- and decaprenyl diphosphate were synthesized when *ω,E,E*-FPP was used as the reaction primer, and when *ω,E,E,E*-GGPP was used, octaprenyl diphosphate was formed at a very low

rate (~1 pmol/mg/min) (Figure 5.3, Panels C and D, respectively).

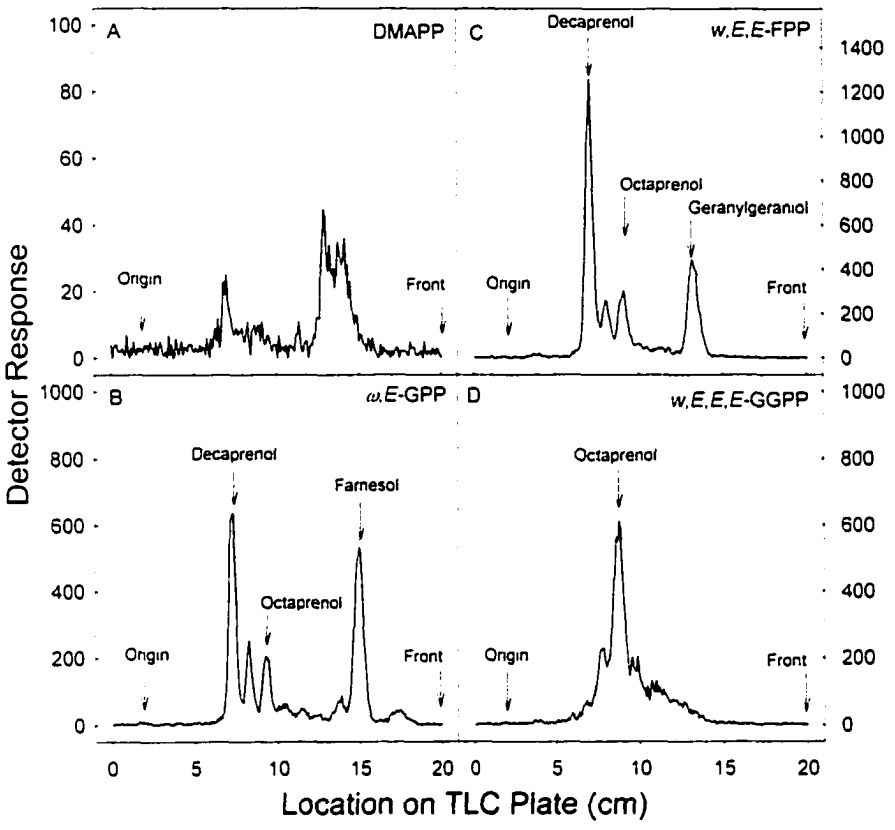

Figure 5.3- TLC analysis of products synthesized by *M. tuberculosis* membranes in the presence of [^{14}C]IPP and DMAPP (Panel A), GPP (Panel B), *ω,E,E*-FPP (Panel C) or *ω,E,E,E*-GGPP (Panel D). The radiolabelled prenyl diphosphates were dephosphorylated with potato acid phosphatase. Equivalent amounts of radioactivity derived from dephosphorylated products were analyzed on $LKC_{18}F$ TLC plates developed in methanol/acetone (8:2, v/v) except in Panel A where all of the radioactivity was loaded. Radioactive spots were located with a Bioscan System 200 Imaging Scanner (Bioscan Inc.) and standard polyprenols were located with an anisaldehyde spray reagent.

Characterization of stereochemistry of enzymatically labeled products- When the relatively small amount of FPP produced by *M. tuberculosis* cytosol incubated with either DMAPP or *ω,E*-GPP as the primer (Figure 5.2) was dephosphorylated and

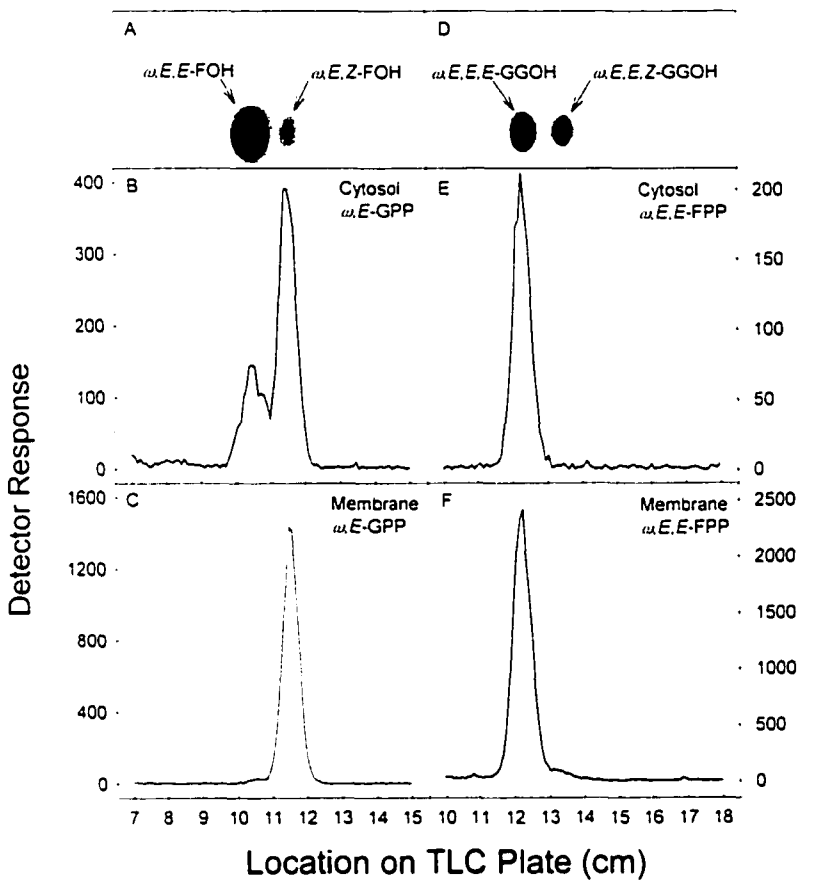

Figure 5.4- Stereochemical analysis of FPP and GGPP enzymatically synthesized by *M. tuberculosis* cytosol or membranes using *ω,E*-GPP (Panels B and C) or *ω,E,E*-FPP (Panels E and F) as the allylic primer. Dephosphorylated products were analyzed on $LKC_{18}F$ TLC plates. The radiolabelled products were scraped and extracted from the gel and applied to a silica gel 60 TLC plate, which was developed in toluene/ethyl acetate (7:3, v/v). Radioactive spots were located with a Bioscan System Imaging Scanner (Bioscan Inc.) and standard polyprenols (Panels A and D) were located with an anisaldehyde spray reagent.

analyzed for stereochemistry by TLC, it was apparent that the *ω,E,Z* configuration was dominant (Figure 5.4, Panel B). Small, but reproducible, amounts of *ω,E,E*-FPP were seen in these reactions. The majority of the radiolabelled GGPP produced when *M. tuberculosis* cytosol was incubated with *ω,E,E*-FPP was identified as *ω,E,E,E*-geranylgeraniol after it had been dephosphorylated (Figure 5.4, Panel E). The farnesol that was produced by dephosphorylation of the radiolabelled products generated by incubation of *M. tuberculosis* membranes with [^{14}C]IPP and *ω,E*-GPP is virtually all *ω,E,Z*-farnesol (Figure 5.4 Panel C). When the geranylgeraniol that was produced by the dephosphorylation of the radiolabelled products generated when *M. tuberculosis* membranes were incubated with *ω,E,E*-FPP as the primer was analyzed for stereoconfiguration the material co-migrated with authentic *ω,E,E,E* geranylgeraniol (Figure 5.4, Panel F).

Isoprenyl diphosphate synthases in mycobacterial cytosol- The major product seen when cytosol was incubated with [^{14}C]IPP and DMAPP, *ω,E*-GPP or *ω,E,E*-FPP was *ω,E,E,E*-GGPP. Interestingly, no radioactive *ω,E*-GPP was synthesized from DMAPP even though *ω,E*-GPP is an obligate intermediate in the synthesis of all of the larger prenyl diphosphates. Similarly only small amounts of *ω,E,E*-FPP were found although it is also an obligate intermediate in the conversion of DMAPP

and/or ω,E-GPP to ω,E,E,E-GGPP. The majority of the FPP synthesized by both the cytosol and membranes was ω,E,Z-FPP, which is unlikely to be an intermediate in the synthesis of ω,E,E,E-GGPP. Since ω,E,E,E-GGPP was the only isomer of GGPP isolated from the cytosolic incubations and both isomers of FPP are water soluble (and hence available in the reaction mixtures) these results suggest that there may be a single multifunctional enzyme synthesizing ω,E,E,E-GGPP from DMAPP in mycobacterial cytosol. If this is the case, the enzyme would be analogous to several eukaryotic ω,E,E-FPP synthases that are also multifunctional. For example, purified and crystallized avian ω,E,E-FPP synthase synthesizes primarily ω,E,E-FPP when incubated with DMAPP as the allylic primer and releases only trace amounts of the ω,E-GPP intermediate[3].

Isoprenyl diphosphate synthases in mycobacterial membranes- The predominant products of reactions using *M. tuberculosis* membranes and ω,E-GPP as the reaction primer were ω,E,Z-FPP and decaprenyl diphosphate, however, when ω,E,E-FPP was used in the reaction the primary products were decaprenyl diphosphate and ω,E,E,E-geranylgeranyl diphosphate.

M. tuberculosis membranes incubated with ω,E,E,E-GGPP synthesized octaprenyl diphosphate (at a very low rate). It is unlikely that the octaprenyl diphosphate synthesized by *M.*

tuberculosis is a precursor of decaprenyl diphosphate since very little decaprenyl diphosphate was synthesized in the presence of *ω,E,E,E*-GGPP but was the primary product when *ω,E*-GPP was the reaction primer. Since it is not possible to assign stereochemistry to molecules to which more than one isoprene unit is added (using the techniques reported here) it was not clear whether the octaprenyl diphosphate synthesized by *M. tuberculosis* membranes when *ω,E*-GPP was used as a primer was identical to the octaprenyl diphosphate synthesized when *ω,E,E,E*-GGPP was used. It is also unclear what the function of the octaprenyl diphosphate is, as it has not been reported as a mannose carrier[1].

When *ω,E,E*-FPP was incubated with the *M. tuberculosis* membranes, decaprenyl diphosphate was synthesized even though *ω,E,Z*-FPP is the precursor for decaprenyl diphosphate (assuming that the decaprenyl phosphate from this organism has the same stereochemistry as decaprenyl phosphate from *M. smegmatis*)[4]. This observation suggests that the enzyme that initiates the additions of IPP to FPP in order to form decaprenyl diphosphate is not strictly specific for one stereoisomer, as reported for other prenyl diphosphate synthases that are capable of utilizing allylic primers of various chain lengths and stereochemistries as substrates[5-8].

Isoprenoid chain elongation in Mycobacterium tuberculosis- *M. tuberculosis* has been shown to contain decaprenyl phosphate with unknown stereochemistry[1]. The Pol-P of *E. coli*, *B. subtilis* and other prokaryotes is *ω,diE,polyZ*-undecaprenyl phosphate[9-11]. Because of the stereochemistry at the *ω*-end of the molecule, the C_{15} precursor of *ω,diE,polyZ*-undecaprenyl phosphate must be *ω,E,E*-FPP. The cell free extracts of *M. tuberculosis* do not synthesize *ω,E,E*-FPP in significant amounts, but *M. tuberculosis* does synthesize *ω,E,Z*-FPP. If *ω,E,Z*-FPP is the intermediate of the decaprenyl phosphate molecule isolated by Takayama[1], then the decaprenyl phosphate from *M. tuberculosis* will have the same stereoconfiguration as the decaprenyl phosphate isolated from *M. smegmatis*[4]. In addition to *ω,E,Z*-FPP, the cell free extracts of *M. tuberculosis* also synthesizes *ω,E,E,E*-GGPP, octaprenyl diphosphate and decaprenyl diphosphate.

C. Isoprenyl Diphosphate Synthase Homologs in *M. tuberculosis*

A second preliminary investigation was used to identify open reading frames in the *M. tuberculosis* H37Rv genome sequence that had amino acid sequence similarity to known *E*- and *Z*-isoprenyl diphosphate synthases. The putative isoprenyl diphosphate synthases were cloned and overexpressed. The protein from the corresponding recombinant was assayed for

isoprenyl diphosphate synthase activity. This strategy led to the identification of three novel isoprenyl diphosphate synthases from *M. tuberculosis* which are the subjects of Chapters 6-8.

Putative isoprenyl diphosphate synthases in the M. tuberculosis genome- The complete DNA sequence of *M. tuberculosis* H37Rv has been reported[12]. The sequences of known isoprenyl diphosphate synthases (*E* and *Z*) were used to identify isoprenyl diphosphate synthases within the genome of *M. tuberculosis*. Five open reading frames were identified that have homology to the *E*-isoprenyl diphosphate synthase family. Three of these contain the GGPP synthase type I motif[13]; the FARM, DDXXD and an aromatic amino acid at -5 to the FARM (Figure 5.5). The remaining two open reading frames with *E*-isoprenyl diphosphate synthase homology had motifs reminiscent of medium chain *E*-isoprenyl diphosphate synthases [13](Figure 5.6). Two open reading frames were identified that have homology to *Z*-isoprenyl diphosphate synthases (Figure 5.7)[14].

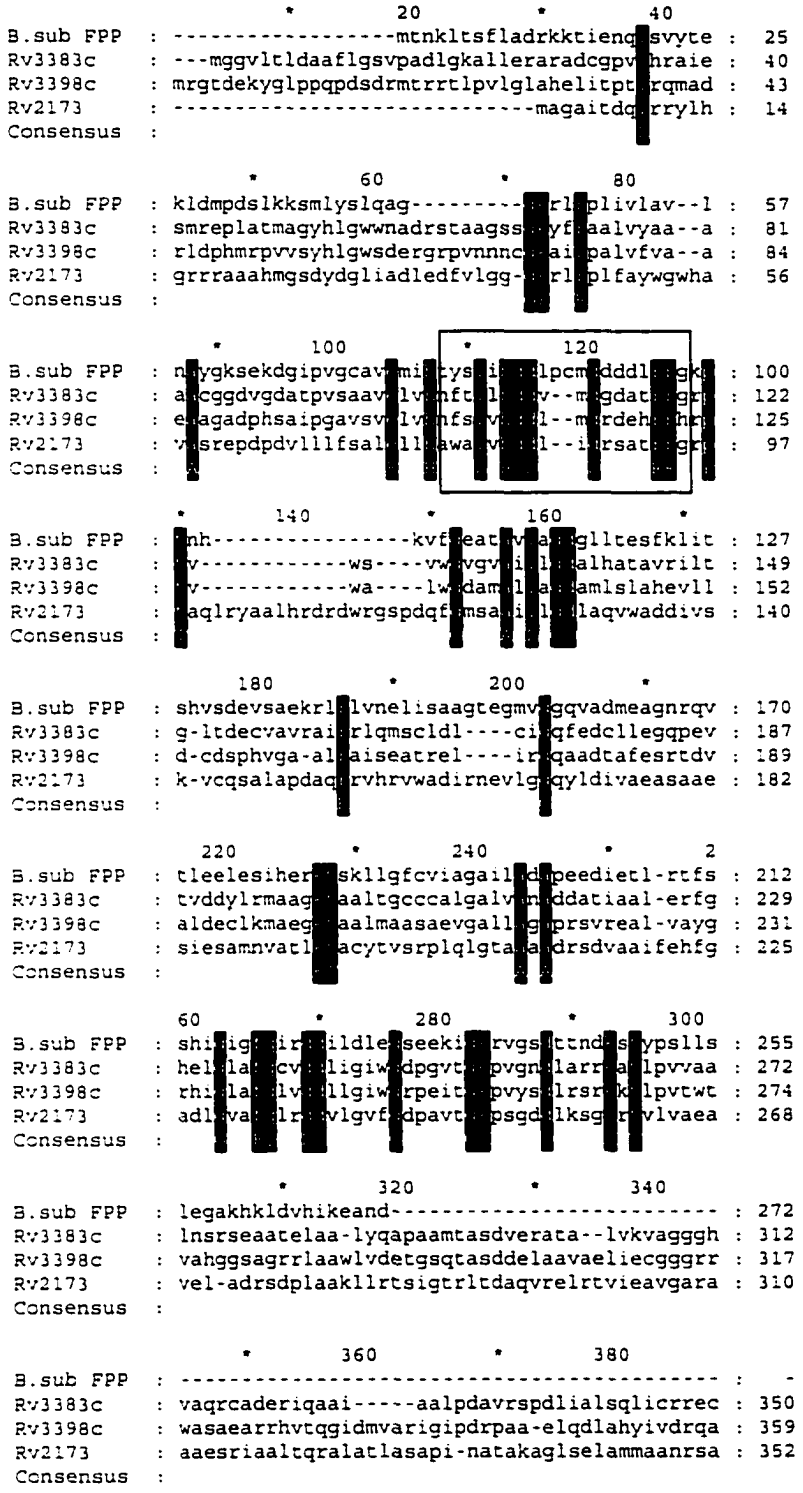

Figure 5.5- Alignment of the FPP synthase from *B. subtilis* (B.sub FPP) with three open reading frames of *M. tuberculosis* H37Rv. Conserved amino acids are highlighted in black. The chain length determining region is boxed. The *M. tuberculosis* homologs do not contain an insertion in the FARM, DDXXD (amino acids 114-120), but they do contain aromatic amino acids in the position -5 to the FARM (amino acid 109).

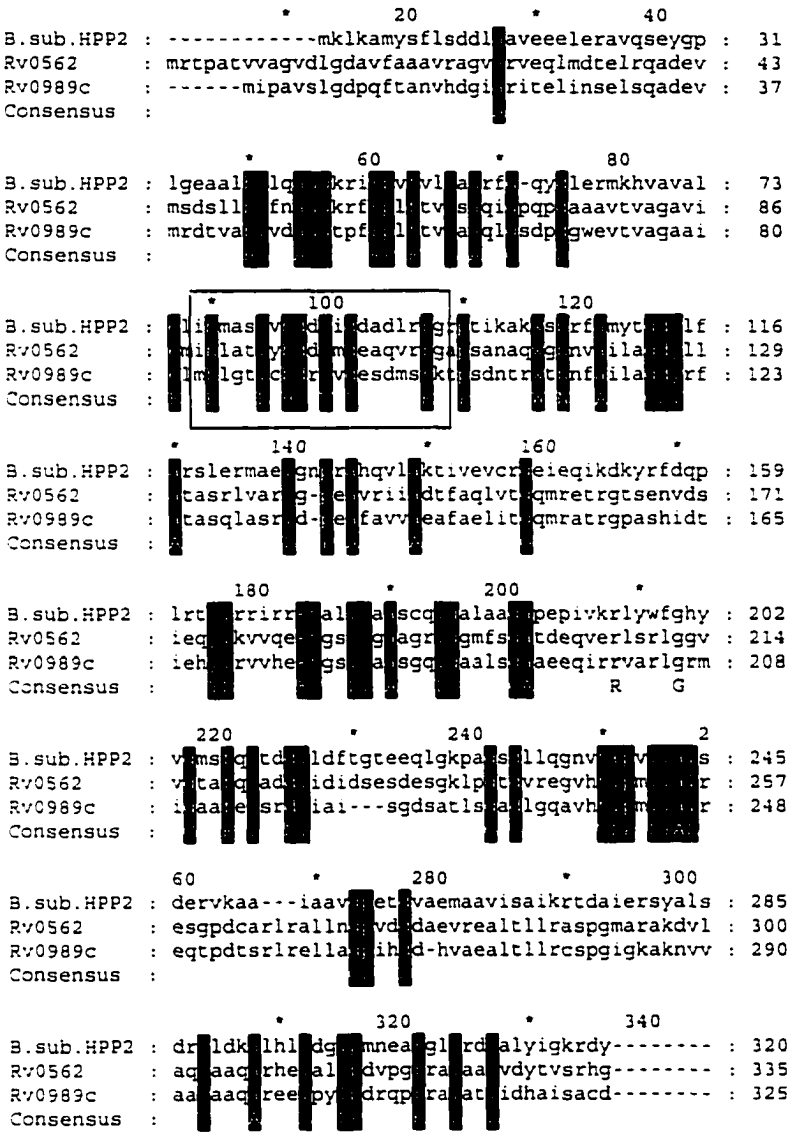

Figure 5.6- Alignment of the heptaprenyl diphosphate synthase (large subunit) from *B. subtilis* (B.sub.HPP2) with two open reading frames from *M. tuberculosis* H37Rv. Conserved amino acids are highlighted in black. The chain length determining region is boxed. Rv0562 contains the DDXXD motif and an isoleucine residue at position -8 before the FARM. This motif is reminiscent of the medium chain isoprenyl diphosphate synthases. Rv0989c does not contain a complete FARM motif, (DRXXD) and it has poor homology to key residues in other conserved regions, however it still has some features similar to *E*-isoprenyl diphosphate synthases.

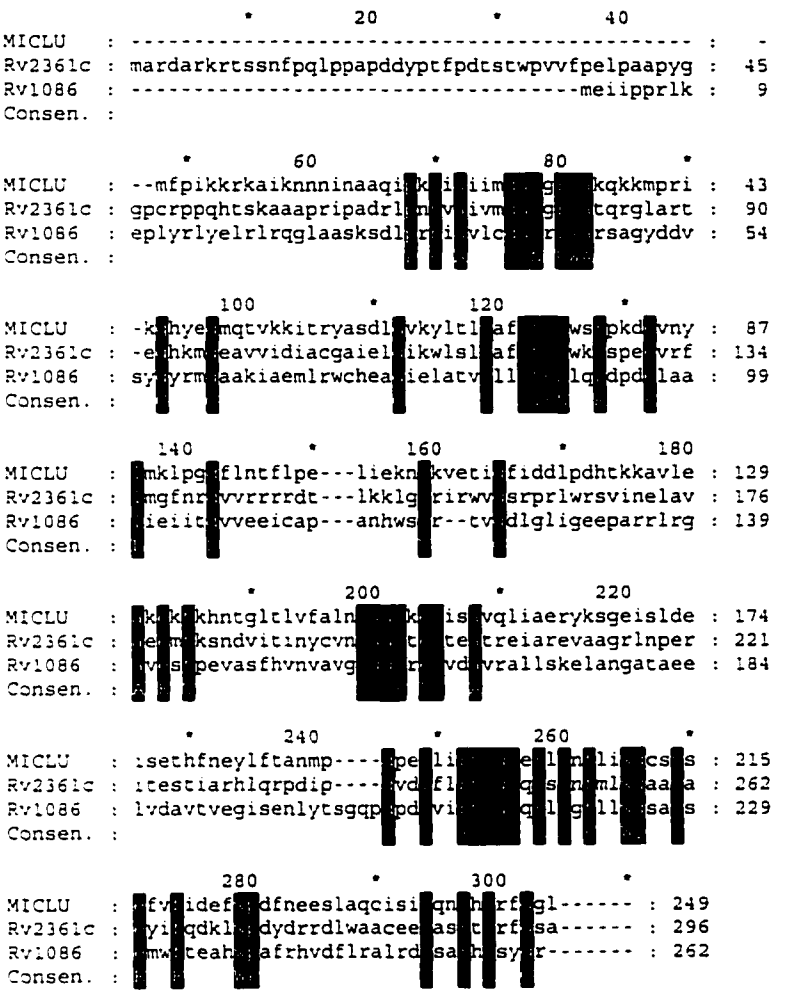

Figure 5.7- The amino acid alignment of undecaprenyl diphosphate synthase from *Micrococcus luteus* (MICLU) and two open reading frames from *M. tuberculosis* H37Rv. Conserved amino acids are highlighted in black.

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Chapter 6.

Identification of a short (C_{15}) chain Z-Isoprenyl diphosphate synthase and a homologous long (C_{50}) chain isoprenyl diphosphate synthase in *Mycobacterium tuberculosis*.

In Chapter 5, open reading frames homologous to known isoprenyl diphosphate synthases were identified in the genome of *M. tuberculosis* H37Rv[1]. In order to determine if the open reading frames encoded functional isoprenyl diphosphate synthases, they were cloned and overexpressed in *M. smegmatis*. Protein from recombinant *M. smegmatis* was assayed for isoprenyl diphosphate synthase activity. The data from the recombinant assays were compared to the data from wildtype *M. smegmatis* isoprenyl diphosphate synthase assays. This chapter discusses the results obtained from the analysis of the two *Z*-isoprenyl diphosphate synthase homologs. The results were presented in Identification of a short (C_{15}) chain *Z*-Isoprenyl diphosphate synthase and a homologous long (C_{50}) chain isoprenyl diphosphate synthase in *Mycobacterium tuberculosis*. (Schulbach, Mark C., Patrick J. Brennan, Dean C. Crick. 2000. Journal of Biological Chemistry. Vol. 273, No. 30 pp.22876-22881)[2]

A. Introduction

The isoprenoid compounds are chemically diverse, with over 23,000 compounds currently characterized[3]. Representative members of

this family of compounds (cholesterol, quinones, carotenoids, polyprenyl phosphates and rubber) display diversity in structure as well as function. Polyprenol phosphate (Pol-P) 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[4-7]. Eubacteria usually contain a single Pol-P molecule (C_{55}) composed of 11 isoprene units; *ω,diE,polyZ*-undecaprenyl-phosphate[8,9]. Mycobacteria are exceptions to this rule. It was demonstrated that *M. smegmatis* possesses two unique Pol-P: 1) a heptaprenyl-phosphate (C_{35}) with four saturated, two *E* and one *Z* double bond[10] and 2) a decaprenyl-phosphate (C_{50}) with one *E* and eight *Z* double bonds[11]. In 1998, Wolucka and de Hoffman isolated a form of heptaprenyl-phosphate that contained four saturated and three *Z* isoprene units from *M. smegmatis*[12]. All of these Pol-P molecules were isolated as mannosyl-1-phosphoryl-polyprenols (Pol-P-Man), and probably have roles in mannan and arabinomannan synthesis[13]. *M. tuberculosis* appears to be more typical of other eubacteria than *M. smegmatis*, as it appears to contain a single Pol-P molecule, decaprenyl phosphate whose stereochemistry has not yet been determined[14].

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-myo-inositol-oligomannosides (PIMs) to give rise to PIM-containing lipomannan and lipoarabinomannan[15]. In addition, heptaprenyl phosphate is found in the form of mycolyl-6-α-D-mannosyl-1-phosphoryl-heptaprenol and may function to carry mature mycolic acids across

the plasma membrane[10]. Arabinosyl-1-phosphoryl-decaprenol is donor of the arabinofuranosyl residue in the arabinan of arabinogalactan, arabinomannan and lipoarabinomannan[11]. 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[13]. Pol-P (probably the decaprenyl-phosphate) also has a role in mycobacterial peptidoglycan synthesis[7;16]. In spite of 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, C_{10})[17]. This and subsequent additions of IPP to the growing allylic diphosphate (to form farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}) 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.

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[3]. Amino acid alignments of these enzymes generated by Chen *et al.* in 1994 and again by Kellogg and Poulter in 1997 have defined five conserved regions, including two aspartic acid rich $(DD(XX)_2D$ motifs[18;19]. However, the resulting consensus sequences have to date been useful in identifying only those enzymes catalyzing *E* double bonds. Enzymes responsible for catalyzing *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*-polyprenyl chain elongation of the allylic substrate *ω,E,E*-FPP[21], the first of such enzymes to have its amino acid sequence determined. As anticipated it had no homology to any of the *E*-isoprenyl diphosphate synthases. Apfel *et al.*[21] 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 cervisiae* has been identified[22;23].

The determination of the complete genome sequence for *Mycobacterium tuberculosis* H37Rv, has allowed for systematic exploration of gene function in this organism[1]. We have identified two open reading frames in the *M. tuberculosis* H37Rv genome, Rv1086 and Rv2361c, whose predicted protein products have

homology to the undecaprenyl diphosphate proteins identified by Shimizu *et al.* and Apfel *et al.*, and homology to the yeast dolichol synthase[20-23]. The open reading frames of the putative *M. tuberculosis* Z-isoprenyl diphosphate synthases were cloned and over-expressed in an attempt to map the pathway for the synthesis of Pol-P.

B. Experimental Procedures

The general experimental procedures were previously discussed in Chapter 4. Those procedures relative to this chapter are: subcellular fractionation, *in vitro* isoprenyl diphosphate synthase assays, enzymatic treatment of reaction products, analysis of radiolabelled product chain length and analysis of radiolabelled product stereochemistry. The unique methods are described below.

Cloning of Isoprenyl Diphosphate Synthases- Open reading frames Rv1086 and Rv2361c in the *M. tuberculosis* H37Rv genome[1] were identified as potential isoprenyl diphosphate synthases based on homology to *M. luteus* undecaprenyl diphosphate synthase[20]. Advanced BLAST searches were performed online at the National Center for Biotechnology Information. Alignments were created using Multalin and Genedoc. The following primers were designed to amplify open reading frame Rv1086 from H37Rv genomic DNA: 5'-GGTA*CATATG*GAGATCATCCCGCCG-3' and 5'-CGTCCTGCG*AAGCTT*TCACCTGCCG-3'. Primers were also designed to amplify open reading frame Rv2361c

from H37Rv genomic DNA: (5'-ATAT*CATATG*GCTAGGGATGCACGG-3' and 5'-CCGGTAGG*AAGCTT*CTAGGCGCTC-3'). *Nde*I and *Hind*III restriction endonuclease sites (nucleotides underlined in above primer sequences) were engineered into the N-terminal and C-terminal primers, respectively. PCR was performed on a Perkin Elmer GeneAmp 2400 PCR System using Vent DNA Polymerase. The PCR products were digested with *Nde*I and *Hind*III and ligated into the mycobacterial expression vector pVV16 (a gift from Dr. Varalakshmi Vissa, Colorado State University), which had been previously digested with the same enzymes. The ligation mixture was electroporated into *E. coli* compentent cells (XL-1 Blue). Cells containing plasmid were selected on LB agar containing kanamycin at a concentration of 20 μg/ml. Purified plasmids were subjected to restriction and sequence analysis. The resulting constructs, named pVV-Rv1086 and pVV-Rv2361c, were electroporated into *M. smegmatis* mc^2155. In addition, pVV16 (without insert) was electroporated into *M. smegmatis* for use as a control. For electroporation, *M. smegmatis* cells were grown to late log phase in LB broth, washed seven times with ice-cold, sterile 10% glycerol and frozen at -70°C in 50 μl aliquots until used. Electroporation was performed at 2.5 volts, 800 ohms, 25 μfarads. Cells were recovered in LB broth for 90 min. and plated on LB agar with kanamycin (20 μg/ml). A single colony was chosen to start a liquid cultures in LB broth with kanamycin (20 μg/ml).

C. Results

Recombinants Rv1086 and Rv2361c increase [^{14}C]IPP incorporation into butanol extractable material- Rv1086 and Rv2361c were cloned into pVV16. In this mycobacterial specific expression vector, the cloned genes are constituitively expressed under the control of the heat shock promoter HSP-60. Protein (membrane or cytosol) from wildtype, empty vector or recombinant strains was assayed for [^{14}C]IPP incorporation into butanol extractable material in the presence of ω,E-GPP or ω,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 ω,E-GPP showed an increase of [^{14}C]IPP incorporation into butanol extractable material when compared to the wildtype cytosolic assays (Table 6.1). However, only the Rv2361c cytosolic assay primed with ω,E,E-FPP showed an increase in [^{14}C]IPP incorporation. Assays of membrane protein revealed similar results (Table 6.1). The Rv2361c recombinant membrane protein was able to utilize both primers more effectively than wildtype membrane protein. The Rv1086 recombinant membrane protein was able to utilize more [^{14}C]IPP than wildtype membrane protein when primed with ω,E-GPP, but not when primed with ω,E,E-FPP. Protein fractions derived from the strains expressing Rv1086 or Rv2361c, had increased isoprenyl diphosphate synthase activity when compared to the corresponding wildtype protein fractions.

TLC analysis reveals that the Rv1086 and Rv2361c recombinants have increased [^{14}C]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. Figures 6.1 and 6.2

Table 6.1. Incorporation of [^{14}C]IPP into allylic diphosphates catalyzed by cytosolic or membrane fractions prepared from wildtype M. smegmatis, or recombinant M. smegmatis.

Allylic Substrate	Cytosolic Activity (pmol/mg/min)			Membrane Activity (pmol/mg/min)		
	WT	Rv1086	Rv2361c	WT	Rv1086	Rv2361c
GPP	298	926	614	432	966	1108
FPP	245	145	476	260	258	487

Cytosol and membrane fractions from wildtype *M. smegmatis*, or recombinant *M. smegmatis*, expressing Rv1086 or Rv2361c, were assayed for [^{14}C]IPP incorporation into allylic diphosphates. Isoprenyl diphosphate synthase activity was assayed in mixtures containing 50 mM MOPS (pH 7.9), 10mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5mM dithiothreitol, 0.3% Triton X-100, 100 μM allylic diphosphate, 30 μM [^{14}C]IPP, and 75 μg protein in a final volume of 50 μl. Reactions were incubated for 20 min. and stopped by addition of 1ml water saturated with NaCl. The products were extracted with n-butanol saturated with water and subjected to scintillation spectrometry. Above abbreviations: GPP *ω,E*-geranyl diphosphate; FPP, *ω,E,E*-farnesyl diphosphate; WT, wildtype.

show the migration of [^{14}C]IPP labeled products from the cytosol and membrane reactions, respectively. Wildtype cytosol primed with ω,E-GPP (Figure 6.1A) primarily produces geranylgeranyl diphosphate (specific activity (s.a.) = 173 pmol/mg/min). The smaller amounts of FPP (s.a. = 69 pmol/mg/min) and heptaprenyl diphosphate (s.a. = 27 pmol/mg/min) may be due to imperfect fractionation of membrane and cytosolic proteins as these products are seen at approximately 2 to 3-fold higher concentration in the membrane assays (compare Figures 6.1A and 6.2A). Wildtype cytosol when primed with ω,E,E-FPP (Figure 6.1B), once again primarily produced geranylgeranyl diphosphate (s.a. = 169 pmol/mg/min). Rv2361c cytosolic assays primed with ω,E-GPP and ω,E,E-FPP (Figure 6.2C and 6.2D) showed an increase in the synthesis of decaprenyl diphosphate (s.a. = 141 pmol/mg/min and 126 pmol/mg/min, respectively) an activity that was almost undetectable in wildtype cytosol. Products corresponding to the calculated migrations of octaprenol and nonaprenol are also present. Rv1086 cytosolic assays primed with ω,E-GPP (Figure 6.1E) showed a 10-fold increase of [^{14}C]IPP incorporation into FPP (s.a. = 714 pmol/mg/min) versus wildtype (s.a. = 69 pmol/mg/min), whereas Rv1086 cytosolic assays primed with ω,E,E-FPP (Figure 6.1F) showed a 3-fold decrease in FPP synthesis (but had approximately equal heptaprenyl diphosphate synthesis) when compared to the wildtype.

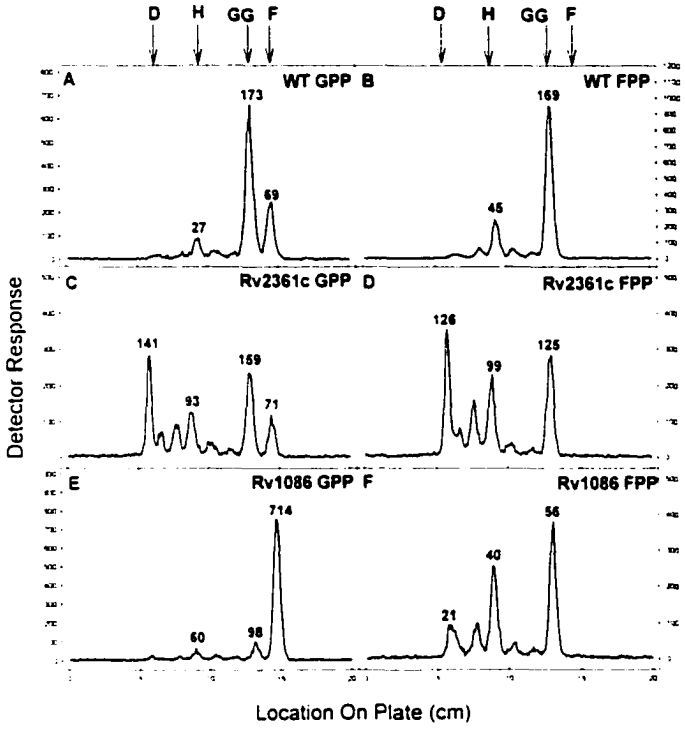

Figure 6.1. TLC analysis of [^{14}C]IPP radiolabelled products synthesized by cytosolic fractions from wildtype or recombinant *M. smegmatis*. Wildtype (WT) or recombinant (expressing Rv2361c or Rv1086) *M. smegmatis* cytosol was assayed in the presence of either geranyl diphosphate (GPP, Panels A, C, E) or farnesyl diphosphate (FPP, Panels B, D, F). Isoprenyl diphosphate synthase activity was assayed and the extracted isoprenyl diphosphates were dephosphorylated with potato acid phosphatase as described in Chapter 4. Equal amounts of radioactivity were spotted on reverse-phase TLC plates. 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. Migration of non-radioactive standards (D, Decaprenol: H, Heptaprenol: GG, Geranylgeraniol: F, Farnesol) are indicated with arrows at the top of the panels. The specific activity of each synthase (pmol/mg/minute) is shown at the top of relevant peaks.

When the products from the wildtype membrane assays primed with *omega, E*-GPP (Figure 2A) are compared to the recombinant membrane assays primed with *omega, E*-GPP (Figures 2C and 2E), there was a significant increase in [^{14}C]IPP incorporation into decaprenyl diphosphate caused by the expression of Rv2361c (s.a. = 443 pmol/mg/min), and into FPP caused by the expression of Rv1086 (s.a. = 701 pmol/mg/min). In the membrane assays primed with ω,E,E-FPP (Figure 6.2, Panels D, E and F), there was a ten-fold increase of [^{14}C]IPP incorporation into decaprenyl diphosphate by the Rv2361c recombinant (s.a. = 205 pmol/mg/min) compared to the corresponding wildtype assay. As expected from the results in Table 1, the Rv1086 membrane assay primed with ω,E,E-FPP did not reveal any increased incorporation when compared to the corresponding wildtype assay.

Rv1086 catalyzes synthesis of ω,E,Z-farnesyl diphosphate- The farnesyl diphosphate created by adding one molecule of [^{14}C]IPP to ω,E-GPP can have two possible stereochemistries, ω,E,E-FPP or ω,E,Z-FPP. It was possible to separate these stereoisomers by silica gel TLC (Figure 6.3, lane 2). The farnesol produced from enzymatically dephosphorylating the products of the Rv1086 membrane assay primed with ω,E-GPP (Figure 6.2E) was recovered from reverse-phase TLC plate and loaded onto silica gel-60 TLC plates. The recovered farnesol migrated along with cold standard ω,E,Z-farnesol, which runs ahead of cold standard ω,E,E-farnesol

(Figure 6.3, lanes 2 and 3). The farnesol produced from enzymatically dephosphorylating the products of the Rv1086 cytosolic assay primed with ω,E-GPP was also in the ω,E,Z-configuration (data not shown).

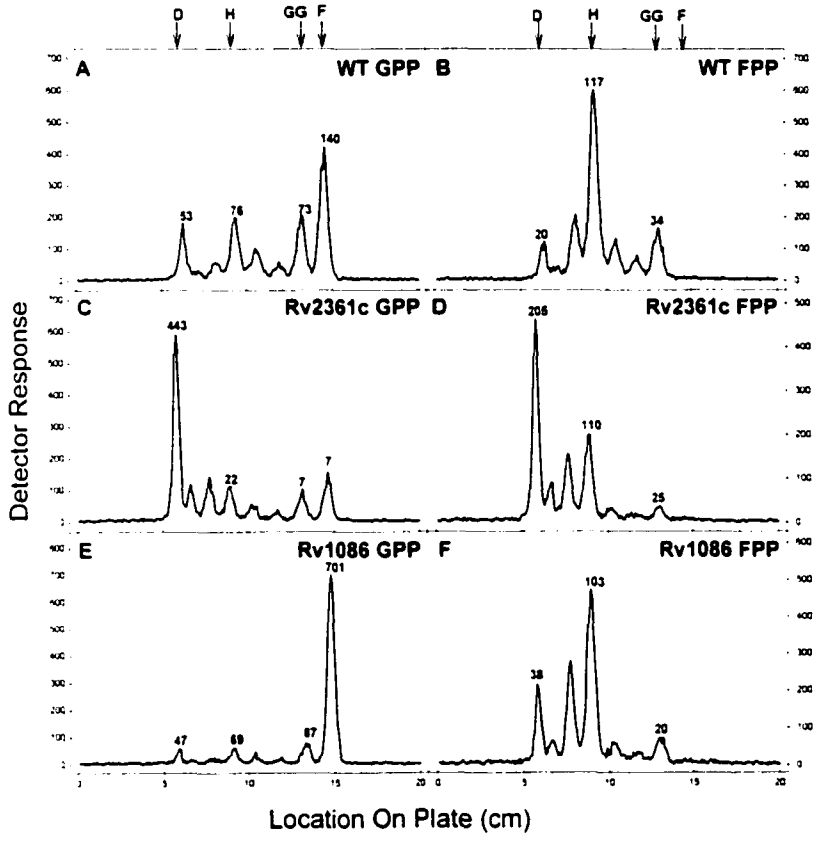

Figure 6.2. *TLC analysis of [^{14}C]IPP radiolabelled products synthesized by membrane fractions from wildtype or recombinant M. smegmatis.* Wildtype (WT) or recombinant (expressing Rv2361c or Rv1086) *M. smegmatis* membrane was assayed in the presence of either geranyl diphosphate (GPP, Panels A, C, E) or farnesyl diphosphate (FPP, Panels B, D, F). Assay conditions and identification of products were as described in Figure 6.1.

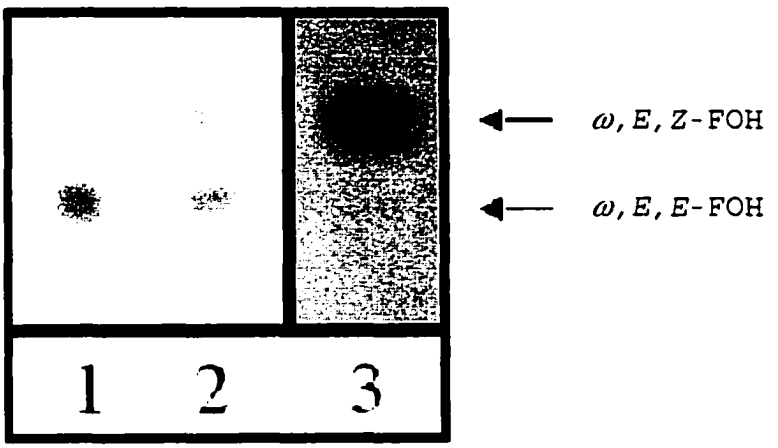

Figure 6.3. *Stereochemical analysis of farnesyl diphosphate enzymatically synthesized by the membranes of recombinant M. smegmatis expressing Rv1086.* Assay conditions are described in Figure 6.1. Assay products were dephosphorylated and subjected to reverse-phase TLC. The radiolabelled material corresponding to farnesol was scraped from the reverse-phase TLC plates and extracted as described in Chapter 4. The recovered farnesol was spotted onto a silica gel 60 TLC plate and developed in toluene:ethyl acetate (7:3, v/v). Cold *ω,E,E*-farnesol was loaded onto lane 1. Mixed isomers of farnesol (*ω,E,E*-farnesol and *ω,E,Z*-farnesol) along with the products of the Rv1086 assay were loaded in lane 2. Lane 3 shows an autoradiogram of lane 2.

D. Discussion

The *M. tuberculosis* open reading frame Rv1086 encodes an *ω,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 synthases in genomic databases. 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[28].

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 presumed allylic diphosphate substrate *in vivo* would be *ω,E,Z*-FPP and each molecule of IPP would be added with *Z*-stereoconfiguration to yield *ω,E,polyZ*-decaprenyl phosphate[24]. Our assays showed that Rv2361c was able to use the allylic primers *ω,E*-GPP and *ω,E,E*-FPP. *ω,E*-GPP may not be used directly for DecaPP synthesis, but instead may be used for *ω,E,Z*-FPP synthesis by the background

wildtype *M. smegmatis* enzymes. *ω,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 (Figure 6.2C) when compared to the wildtype membrane assay (Figure 6.2A). On the other hand, *ω,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[9,25,26]. 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[20], had no homology to the *E*-isoprenyl diphosphate synthases[18,19]. It did however contain an aspartate rich $DD(XX)_2D$ motif reminiscent of the *E*-isoprenyl diphosphate synthases. Shimizu *et al.* suggested that this motif may represent the diphosphate binding site. The signature motif (Figure 6.4, 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, Rv1086 along with all of the biochemically assayed Z-isoprenyl diphosphate synthases known at this time [undecaprenyl diphosphate synthases from *M. luteus*, *Escherichia coli*, *Haemophilis influenzae*, *Streptococcus pneumoniae* and dolichol synthase from *Saccharomyces cervisiae* (Figure 6.4)]. This alignment demonstrates that there are several regions of amino acid sequence conservation among the *Z*-isoprenyl diphosphate synthases. The amino acid sequence conservation can be roughly distributed into four regions, which we have designated A through D (to avoid confusion with the five conserved regions (I-V) in *E*-isoprenyl diphosphate synthases). Region B contains a high degree of residues with charged and uncharged polar sidechains, which could hypothetically aid in binding of the diphosphate moiety in the active site. Region B also contains conserved amino acids with large aromatic sidechains such as tryptophan and phenylalanine (Figure 6.4, 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

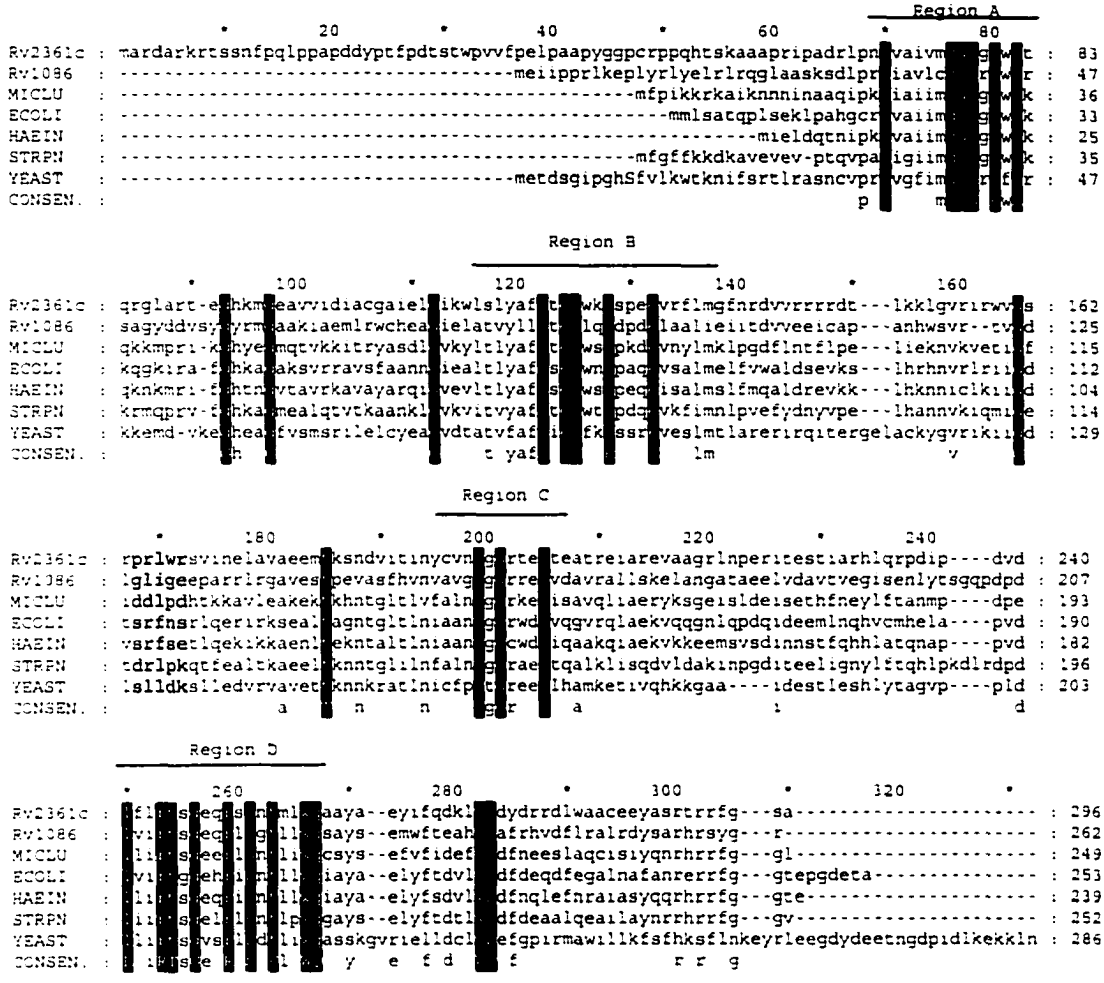

Figure 6.4. *Multiple sequence alignment of open reading frames Rv2361c and Rv1086 of Mycobacterium tuberculosis H37Rv genome sequence with known Z-isoprenyl diphosphate synthases.* Conserved amino acids are highlighted in black. Conserved regions are indicated with bars A-D. The $DD(XX)_2D$ motif in MICLU (amino acids 168-172, grey highlight) does not exist in other homologs. MICLU, *Micrococcus luteus* undecaprenyl diphosphate synthase, SWISS-PROT O82827; ECOLI, *Escherichia coli* undecaprenyl diphosphate synthase, SWISS-PROT Q47675; HAEIN, *Haemophilis influenzae* undecaprenyl diphosphate synthase, SWISS-PROT P44938; STRPN, *Streptococcus pneumoniae* undecaprenyl diphosphate synthase (18); and YEAST, *Saccharomyces cervisiae dehydrodolichyl diphosphate synthetase*, SWISS-PROT P35196. Alignment was generated by Multalin and edited in Genedoc.

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 carboxy 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 fraction of *M. tuberculosis* H37Rv, the subcellular location of Rv1086 is more ambiguous as activity can be found in both the cytosolic and membrane fractions[27].

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 *Micrococcus luteus* undecaprenyl diphosphate synthase[28]. It would be of great interest to determine the quaternary structure of the mycobacterial enzymes as they could reveal structural requirements for chain length determination. We have initiated purification of these enzymes.

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Chapter 7.

Purification, Enzymatic Characterization and Inhibition of the *Z*-Farnesyl Diphosphate Synthase from *Mycobacterium tuberculosis*.

Chapter 6 described the identification of two *Z*-isoprenyl diphosphate synthases from *M. tuberculosis*. The short chain *Z*-isoprenyl diphosphate synthase (encoded by Rv1086) catalyzes the first committed step for the synthesis of decaprenyl phosphate, whose role in cell wall biosynthesis has been discussed (Chapter 1). This chapter describes the purification, characterization and inhibition of the *Z*-farnesyl diphosphate synthase from *M. tuberculosis*. The results have been submitted to the Journal of Biological Chemistry in a manuscript entitled Purification, Enzymatic Characterization and Inhibition of the *Z*-Farnesyl Diphosphate Synthase from *Mycobacterium tuberculosis* (Schulbach, Mark C., Sebabrata Mahapatra, Marco Macchia, Silvia Barontini, Chiara Papi, Filippo Minutolo, Simone Bertini, Patrick J. Brennan and Dean C. Crick).

A. Introduction

Isoprenyl diphosphate synthases catalyze the condensation of an allylic diphosphate with isopentenyl diphosphate (IPP, C_5) via an electrophilic alkylation reaction to produce longer allylic diphosphates[1,2]. Chain elongation continues until a physiologically appropriate chain length is reached, at which

time the molecule may undergo further modifications (dephosphorylation, cyclization, or head to head condensation reactions). Polyprenyl phosphate (Pol-P), is formed by dephosphorylation of an allylic prenyl diphosphate chain. The predominant form of prokaryotic Pol-P is *ω,diE,polyZ*-undecaprenyl phosphate (C_{55}), however, there are documented exceptions in *Paracoccus denitrificans*[3] and in *Mycobacterium* spp.[4-8] *Mycobacterium smegmatis* contains heptaprenyl diphosphate[8] (C_{35}, four saturated, three *Z* double bonds) and decaprenyl diphosphate[5] (C_{50}, *ω*, one *E* and eight *Z* double bonds), while *Mycobacterium tuberculosis* contains only decaprenyl phosphate[6]. Although the stereochemistry of decaprenyl phosphate from *M. tuberculosis* has not been determined, our enzymatic studies suggest that it has similar stereochemistry to decaprenyl phosphate from *M. smegmatis*[9].

Pol-P is central to prokaryotic cell wall synthesis as a sugar carrier, and it has been reported that the levels of Pol-P may be the rate limiting step for *in vivo* cell wall synthesis[10-13]. Our laboratory has shown that Pol-P is instrumental in the synthesis of each component of the covalently linked peptidoglycan-arabinogalactan-mycolic acid cell wall core of mycobacteria, and other non-covalently associated macromolecules such as lipomannan and lipoarabinomannan[5;14;15]. The importance of Pol-P is also demonstrated *in vivo* by the fact that *M. tuberculosis*[16] (and other *Mycobacterium* spp.; authors) are sensitive to the antibiotic bacitracin, which specifically bind isoprenyl diphosphates[17],

intermediates in Pol-P synthesis inhibiting both chain elongation and dephosphorylation reactions.

Evolutionarily, there appears to be two independent families of isoprenyl diphosphate synthases, based on the type of stereochemistry (*E* or *Z*) introduced at the products' new double bond. *E*-isoprenyl diphosphate synthases are capable of catalyzing the chain elongation of a range of substrates, the smallest one being dimethylallyl diphosphate (DMAPP, C_5) with IPP to form ω,E-geranyl diphosphate (ω,E-GPP, C_{10}). Other short chain isoprenyl diphosphates, [ω,E,E-farnesyl diphosphate (ω,E,E-FPP, C_{15}), ω,E,E,E-geranylgeranyl diphosphate (ω,E,E,E-GGPP, C_{20})] are generated by a similar mechanism with additional molecules of IPP. Medium chain *E*-isoprenyl diphosphate synthases use the short chain products as allylic substrates to produce compounds that are C_{30} to C_{50} in length. Medium chain *E*-isoprenyl diphosphate synthases are homologous to the short chain *E*-isoprenyl diphosphate synthases, as both types contain two signature aspartate motifs [$DD(XX)_{1-2}D$].

Thus far, only seven protein sequences have been biochemically correlated with *Z*-isoprenyl diphosphate synthase activity. These include undecaprenyl diphosphate synthases from *Micrococcus luteus, Escherichia coli, Haemophilus influenzae, Streptococcus pnuemoniae;* the dolichol synthase from *Saccharomyces cervisiae;* and the decaprenyl diphosphate synthase and the farnesyl

diphosphate synthase from *M. tuberculosis*[9]. Prior to the identification of the *Z*-farnesyl diphosphate (*Z*-FPP) synthase from *M. tuberculosis*, all known *Z*-isoprenyl diphosphate synthases utilized ω,E,E-FPP or ω,E,E,E-GGPP as the allylic substrate, added multiple units of isopentenyl diphosphate and released long chain (C_{45} and greater) isoprenyl diphosphate molecules with mixed stereochemistry[2].

The crystal structure of the short chain *E*-isoprenyl diphosphate synthase (avian FPP synthase) has been determined[18], and mutagenesis studies have been performed[19-21] providing a solid understanding of how the active site determines chain length of the product[22,23]. However, little is known about *Z*-isoprenyl diphosphate synthases. We have purified and enzymatically characterized the short chain *Z*-isoprenyl diphosphate synthase from *M. tuberculosis* that catalyzes the first committed step for the synthesis of decaprenyl diphosphate, a molecule whose role in cell wall synthesis is strictly essential.

B. Experimental Procedures

General procedures were described in Chapter 4. Among those pertaining to this chapter are: *In vitro* isoprenyl diphosphate synthase assays, enzymatic treatment of reaction products, analysis of radiolabelled product chain length and analysis of radiolabelled product stereochemistry. The chapter specific methods are described below.

Enzymatic Assays and Product Characterization- Assays were done under conditions that were linear for time and protein concentration. In the metal ion dependence studies, the endogenous divalent cations were removed by incubating the enzyme preparation with Bio-Rex 70 minus 400 mesh (sodium form, Bio Rad) on ice for 20 min. The enzymatic activity was reduced to a basal level, but it was not completely abolished, indicating that residual divalent cations were present. To determine which divalent cations supported activity, $MgCl_2$, $ZnCl_2$, $CaCl_2$ or $MnCl_2$ were added to the assay mixtures at the indicated concentrations. In a separate experiment, 10 mM EDTA was added. In order to study the pH dependence of *Z*-FPP synthase activity, a broad range buffer comprised of 250 mM Tris-HCl, 125 mM MES and 125 mM acetic acid was used. The pH was adjusted with tetraethylammonium hydroxide.

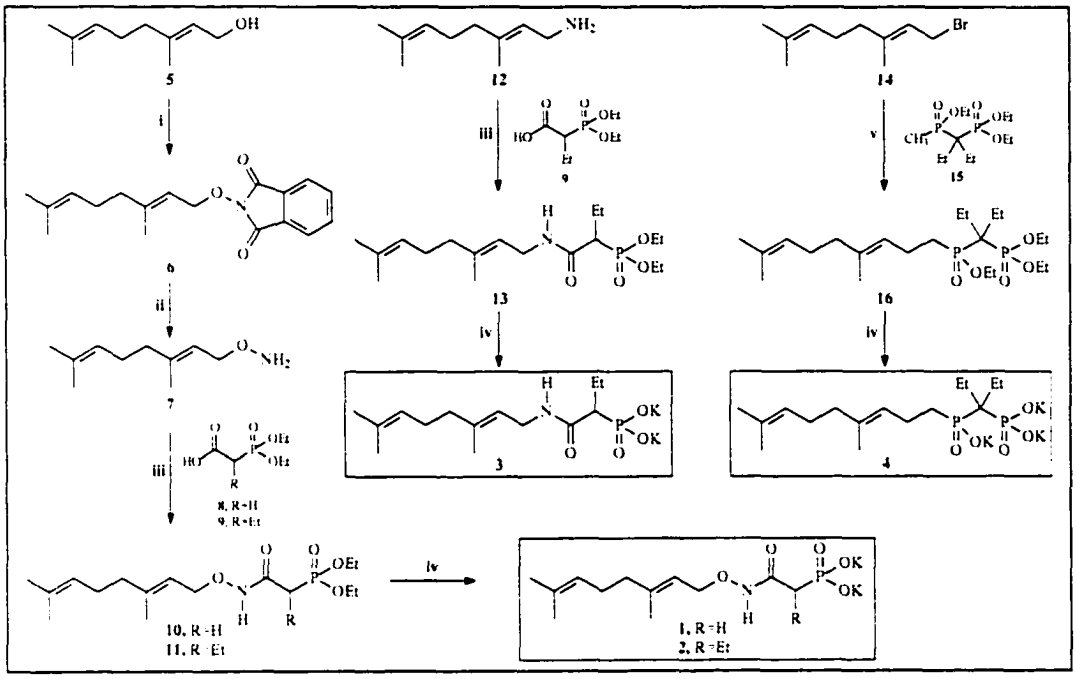
Preparation of Sus scrofa E-FPP Synthase- Pig brain cytosol was prepared by homogenizing pig grey matter in 10 mM HEPES (pH 7.4) and 0.25 M sucrose using 18 passes of a Dounce homogenizer. The homogenates were centrifuged at 9000 g for 15 min at 4°C. The supernate was decanted and centrifuged at 142,000 g for 1 h. The supernate was decanted, divided into 1 ml aliquots, stored at -70°C and used as enzyme source for *E*-FPP synthase assays. The protein was estimated with a BCA protein assay kit (Pierce).

Materials- Citronellyl diphosphate, *ω,E,E*-farnesyl diphosphate and *ω,E*-geranyl diphosphate were synthesized as described by Davisson *et al*[24]. *ω,Z*-Neryl diphosphate was a gift from Drs. J.S. Rush and C.J. Waechter (University of Kentucky).

Synthesis of ω,E-GPP analogs- The syntheses (Refer to Scheme 7.1) were performed by Marco Macchia, Silvia Barontini, Chiara Papi, Filippo Minutolo and Simone Bertini at the Universitá di Pisa, Dipartimento di Scienze Farmaceutiche, Via Bonanno, Pisa, Italy.

All oxygen- and water- sensitive reactions were performed under dry argon atmosphere. ^{1}H-NMR spectra of all compounds were obtained with a Varian Gemini 200 operating at 200 MHz in ca. 2% solution of $CDCl_3$ or D_2O, using Me_4Si or $Me_3Si(CH_2)_3SO_3Na$ as the internal standard. Column chromatographies were performed using 230-400 mesh silica gel (Merck) or reverse phase silica gel (Macherey-Nagel Polygosil® 60-4063 C_{18}). Mass spectra were recorded on a VG 70-25S mass spectrometer or a HP-5988 A spectrometer. Reagents and solvents were purchased from Aldrich or Fluka.

(E)-N-(3,7-dimethyl-2,6-octadienyloxy)phthalimide ***(6)*** — *ω,E*-geraniol **5** (2.0 g, 13 mmol) was added to a solution of *N*-hydroxyphthalimide (2.12 g, 13 mmol), triphenylphosphine (3.42 g,



Scheme 7.1. *Synthesis of ω,E-Geranyl Diphosphate Analogs, Compounds* ***1-4***. Reagents and conditions: i) *N*-hydroxyphthalimide, diethyl azodicarboxylate, triphenylphosphine, anhydrous tetrahydrofuran, 18 h, room temperature; ii) NH_2NH_2, ethanol, 18 h, room temperature; iii) 1-hydroxybenzotriazole, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrocloride, anhydrous tetrahydrofuran, 18 h, room temperature; iv) a. Bromotrimethylsilane, 2,4,6-collidine, dichloromethane, 18 h, room temperature; b. KOH 1N, 3 h, room temperature; v) Butyl lithium 1.6 M hexane solution, anhydrous tetrahydrofuran, 1 h, -78°C.

13 mmol) and diethyl azodicarboxylate (2.25 ml, 14.3 mmol) in anhydrous tetrahydrofuran (50 ml). After stirring for 18 h at room temperature, the solvent was evaporated and the residue was purified on silica gel, eluting with CH_2Cl_2-hexane (6:4). The appropriate fractions were combined and evaporated to give the intermediate **6** (3.19 g, 82%) as a white solid. Elemental analysis of compound **6** was consistent with theoretical values to within ±0.4%. Mp 89-91 °C (uncorrected); ^{1}H-NMR ($CDCl_3$) δ 1.57 (s, 3H, CH_3), 1.64 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 1.93-2.18 (m, 4H, 2×CH_2), 4.72 (d, 2H, *J*=8 Hz, CH_2), 5.01 (br, 1H, CH), 5.53 (t, 1H, *J*=8 Hz, CH), 7.60-7.85 (m, 4H, Ar); Anal. for $C_{18}H_{21}NO_3$: C, 71.93 H, 7.15; N, 4.59. Found: C, 72.15; H, 7.02; N, 4.68.

(E)-3,7-Dimethyl-2,6-octadienyl-1-oxyamine (**7**) — Hydrazine monohydrate (0.69 ml, 14.22 mmol) was added to a solution of **6** (2.13 g, 7.11 mmol) in ethanol (200 ml) and the resulting mixture was stirred at room temperature for 18 h. After filtration of the white solid formed, the solution was evaporated and the resulting crude residue was extracted with diethyl ether (3×100 ml). The diethyl ether was removed by evaporation to give **7** (1.09 g, 91%) as an oil, which was used for the next reactions without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.60 (s, 3H, CH_3), 1.68 (s, 6H, 2×CH_3), 1.96-2.18 (m, 4H, 2×CH_2), 4.17 (d, 2H, *J*=6.4 Hz, CH_2), 5.05 (m, 1H, CH), 5.31 (t, 1H, *J*=6.4 Hz, CH); MS m/e 170 $(M+H)^+$.

*2-(Diethylphosphono)butyric acid (**9**)* — A solution containing KOH (2.35 g, 42 mmol), absolute ethanol (7 ml) and water (3 ml) was added dropwise to triethyl 2-phosphonobutyrate (9.9 ml, 42 mmol) and the resulting mixture was stirred at room temperature for 24 h. The solvents were removed under reduced pressure and the solid residue was triturated with diethyl ether (3x130 ml), which was discarded. The residue was dissolved in water (30 ml) and acidified to pH 1 with HCl 6 M. The solution was then saturated with solid NaCl and extracted with CH_2Cl_2 (3x25 ml). The organic phase was dried and evaporated to give intermediate **9** (8.5 g, 90%) as an oil. ^{1}H-NMR ($CDCl_3$) δ 1.01 (t, 3H, *J*=7.2 Hz, CH_3CH_2), 1.32 (t, 6H, *J*=7.2 Hz, 2xCH_3CH_2), 1.53-2.15 (m, 2H, CH_3CH_2), 2.87 (dt, 1H, *J*=7.2, 22 Hz, CH), 3.92-4.38 (m, 4H, 2xCH_3CH_2); MS m/e 225 $(M+H)^+$.

*Diethyl (E)-{2-oxo-2-[[(3,7-dimethyl-2,6-octadienyl)-oxy] amino] ethyl}phosphonate (**10**) and diethyl (E)-1-[[(3,7-methyl-2,6-octadienyl)oxy]aminocarbonyl]propyl phosphonate (**11**)* — A solution of compound **7** (0.340 g, 2.03 mmol), **8**[25] or **9** (2.23 mmol), and 1-hydroxybenzotriazole (0.410 g, 3.04 mmol) in anhydrous tetrahydrofuran (17 ml) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.470 g, 2.44 mmol). The mixture was stirred at room temperature for 8 h, and the solvent evaporated under reduced pressure. The residue was purified by silica gel column chromatography, eluting with

ethyl acetate-hexane in a ratio of 2:1 in the case of **10** and in a ratio of 1:1 in the case of **11**. The appropriate fractions were combined, evaporated, and pump-dried to give the appropriate intermediate **10** or **11** as an oil. **10** (0.493 g, 70%) ^{1}H-NMR ($CDCl_3$) δ 1.32 (t, 6H, *J*=7.2 Hz, 2×$C\underline{H}_3CH_2$), 1.59 (s, 3H, CH_3), 1.70 (s, 6H, 2×CH_3), 1.93-2.18 (m, 4H, 2×CH_2), 2.82 (d, 2H, *J*=21 Hz, CH_2P), 4.08 (q, 4H, *J*=7.2 Hz, 2×$CH_3C\underline{H}_2$), 4.42 (d, 2H, *J*=7.2 Hz, CH_2), 5.04 (m, 1H, CH), 5.37 (t, 1H, *J*=7.2 Hz, CH); MS (FAB$^-$) m/e 348 $(M+H)^-$. **11** (0.530g, 69%) ^{1}H-NMR ($CDCl_3$) δ 0.99 (t, 3H, *J*=7.2 Hz, $C\underline{H}_3CH_2$), 1.28 (t, 3H, *J*=7.2 Hz, $C\underline{H}_3CH_2$), 1.30 (t, 3H, *J*=7.2 Hz, $C\underline{H}_3CH_2$), 1.59 (s, 3H, CH_3), 1.69 (s, 6H, CH_3), 1.85 -2.21 (m, 6H, 2×CH_2 + $CH_3C\underline{H}_2$), 2.55 (dt, 1H, *J*=7.2, 22 Hz, CH), 3.91-4.27 (m, 4H, 2x$CH_3C\underline{H}_2$), 4.41 (d, 2H, *J*=7.2 Hz, CH_2), 5.07 (br, 1H, CH), 5.38 (t, 1H, *J*=7.2 Hz, CH); MS (FAB$^-$) m/e 376 $(M+H)^-$.

*Dipotassium salt of (E)-[2-oxo-2-[[(3,7-methyl-2,6-octadienyl)-oxy]amino]ethyl] phosphonic acid (**1**)* — Bromotrimethylsilane (0.790 ml, 6 mmol) was added to a stirred solution of compound **10** (0.416 g, 1.2 mmol) and 2,4,6-collidine (0.316 ml, 2.4 mmol) in anhydrous CH_2Cl_2 (12 ml); the resulting mixture was stirred at room temperature for 18 h. After evaporation of the solution, the residue was treated with an aqueous solution of KOH (1N, 9 ml) and then stirred at room temperature for 3 h. The solution was evaporated and the resulting crude residue was purified by column chromatography on reverse phase silica gel, by eluting with

methanol-water (1:4) and collecting 2 ml fractions. The appropriate fractions were combined, evaporated, lyophilized, and pump-dried to give **1** (0.313 g, 71%) as a very hygroscopic white lyophilate: ^{1}H-NMR (D_2O) δ 1.58 (s, 3H, CH_3), 1.64 (s, 3H, CH_3), 1.68 (s, 3H, CH_3), 2.05-2.15 (m, 4H, 2×CH_2), 2.40 (d, 2H, *J*=19 Hz, CH_2P), 4.38 (d, 2H, *J*=7.2 Hz, CH_2), 5.16 (br, 1H, CH), 5.39 (t, 1H, *J*=7.2 Hz, CH); MS (FAB$^+$) m/e 368 (M+H)$^+$.

*Dipotassium salt of (E)-1-[[(3,7-methyl-2,6-octadienyl)-oxy]-aminocarbonyl]propyl phosphonic acid (**2**)* — Compound **2** was prepared following the experimental procedure reported for **1**. **2** (0.322 g, 68%) ^{1}H-NMR (D_2O) δ 0.87 (t, 3H, *J*=7.2 Hz, $C\underline{H}_3CH_2$), 1.62 (s, 3H, CH_3), 1.69 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 2.01-2.29 (m, 6H, 2×CH_2 + $CH_3C\underline{H}_2$), 2.41-2.62 (m, 1H, CH), 4.40 (d, 2H, *J*=7.2 Hz, CH_2), 5.18 (br, 3H, 3×CH), 5.40 (t, 1H, *J*=7.2 Hz, CH); MS (FAB$^+$) m/e 396 (M+H)$^+$.

*Diethyl (E)-1-[(3,7-methyl-2,6 -octadienyl)aminocarbonyl]propyl phosphonate. (**13**)* — A solution of *ω,E*-geranylamine **12** (0.370 g, 2.42 mmol), was treated with **9** (0.600 g, 2.66 mmol) to produce **11** following the experimental procedure reported for **10**. The resulting crude residue was purified on silica gel, eluting with ethyl acetate-hexane (2:3). The appropriate fractions were combined, evaporated, and pump-dried to give intermediate **13** (0.770g, 88%) as an oil; ^{1}H-NMR ($CDCl_3$) δ 1.00 (t, 3H, *J*=7.2 Hz,

$C\underline{H}_3CH_2$), 1.30 (t, 6H, *J*=7.2 Hz, 2x$C\underline{H}_3CH_2$), 1.59 (s, 3H, CH_3), 1.66 (s, 3H, CH_3), 1.69 (s, 3H, CH_3), 1.85-2.25 (m, 6H, 2xCH_2 + $CH_3C\underline{H}_2$), 2.72 (dt, 1H, *J*=7.2, 22 Hz, CH), 3.81 (d, 2H, *J*=7.2 Hz, CH_2), 4.05 (q, 4H, *J*=7.2 Hz, 2x$CH_3C\underline{H}_2$), 5.07 (br, 3H, 3xCH), 5.18 (t, 1H, *J*=7.2 Hz, CH); MS (FAB$^+$) m/e 360 $(M+H)^+$.

*Dipotassium salt of (E)-1-[(3,7-methyl-2,6-octadienyl)-amino-carbonyl]propyl phosphonic acid (**3**)* — Compound **3** was prepared following the experimental procedure reported for **1**. In this case, the resulting crude residue was purified by column chromatography on reverse-phase silica gel, eluting with methanol-water in 2:3 ratio. **3** (0.409g, 90%) ^{1}H-NMR (D_2O) δ 0.87 (t, 3H, *J*=7.2 Hz, $C\underline{H}_3CH_2$), 1.63 (s, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.70 (s, 3H, CH_3), 1.99-2.18 (m, 6H, 2xCH_2 + $CH_3C\underline{H}_2$), 2.22-2.49 (m, 1H, CH), 3.41 (d, 2H, *J*=7.2 Hz, CH_2), 5.12 (br, H, CH), 5.23 (t, 1H, *J*=7.2 Hz, CH); MS (FAB$^+$) m/e 380 $(M+H)^+$.

*Diethyl (E)-1- ethyl -1-[(4,8-dimethyl-3,7-nonadienyl)-hydroxy-phosphoryl]propyl phosphonate (**16**)* — Butyllithium (2.85 ml of 1.6 M hexane solution, 4.56 mmol) was added dropwise to a stirred solution of diethyl 1-[ethoxy(methyl)phosphinoyl]-1-ethylpropylphosphonate **15** [26] (1.200 g, 3.81 mmol), in anhydrous tetrahydrofuran (15 ml), cooled at -78 °C under an argon atmosphere. After stirring for 1 h at -78 °C, *ω,E*-geranyl bromide **14** (0.990 g, 4.56 mmol) was added dropwise and the mixture was

stirred for an additional 1 h at -78 °C. The reaction was quenched with acetic acid (0.546 g, 9.12 mmol), diluted with CH_2Cl_2 (60 ml) and washed with brine. The organic phase was dried and evaporated under reduced pressure. The residue was purified by silica gel column chromatography, eluting with ethyl acetate-hexane (1:3). The appropriate fractions were combined, evaporated, and pump-dried to give the intermediate **16** (0.617 g, 36%) as an oil: ^{1}H-NMR ($CDCl_3$) δ 0.85-1.46 (m, 15H, 5×C<u>H</u>$_3$CH$_2$), 1.59 (s, 3H, CH_3), 1.61 (s, 3H, CH_3), 1.69 (s, 3H, CH_3), 1.82-2.36 (m, 12H, 6×CH_2), 3.92-4.35 (m, 6H, 3×CH$_3$C<u>H</u>$_2$), 4.98-5.19 (m, 2H, 2×CH); MS (FAB$^-$) m/e 451 (M+H)$^-$.

*Tripotassium salt of (E)-1-ethyl-1-[(4,8-dimethyl-3,7-nonadienyl)hydroxyphosphoryl] propyl phosphonic acid (**4**)* — Compound **4** was prepared following the experimental procedure reported for **3**. **4** (0.351 g, 61%) ^{1}H-NMR (D_2O) δ 0.92 (t, 6H, *J*=7.2 Hz, 2x<u>CH</u>$_3$CH$_2$), 1.48 (s, 3H, CH_3), 1.50 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 1.59-2.15 (m, 12H, 6×CH_2), 5.02-5.18 (m, 2H, 2×CH); MS (FAB$^-$) m/e 481 (M+H)$^-$.

Purification of Z-FPP synthase- Open reading frame Rv1086[27] was initially cloned into a commercially available protein expression vector pTBY2 (New England Biolabs Inc.). However, due to the apparent toxic effects of the Rv1086 fusion protein to the *E. coli* cells harboring the plasmid, the coding sequence that

encoded the complete insert/fusion was moved into a mycobacterial expression vector for expression in *M. smegmatis*. The following primers were designed to amplify open reading frame Rv1086 from *M. tuberculosis* H37Rv genomic DNA: 5' GGTA*CATATG*GAGAT CATCCCCCCGCG-3' and 5'-GTCCTGCGCTA*GGGCCC*CCTGCCGTAGCTG-3'. *Nde*I and *Sma*I restriction endonuclease sites were engineered into the above primer sequences respectively (underlined). The native stop codon was removed from the C-terminal primer to allow for an in-frame fusion with the intein/chitin binding domain coded in the vector pTBY2. PCR was performed on a Perkin Elmer GeneAmp 2400 PCR System using Vent DNA Polymerase (New England Biolabs). The PCR products were digested with the restriction endonucleases *Nde*I and *Sma*I, then ligated into pTBY2, which had been previously digested with the same enzymes. The ligation mixture was electroporated into *E. coli* compentent cells (XL-1 Blue, Stratagene). Cells containing plasmid were selected on LB agar (EM Science) containing ampicillin at a concentration of 100 µg/ml. Purified plasmid was subjected to restriction and sequence analysis. The resulting construct, pTBY2-Rv1086, was digested with the restriction endonucleases, *Nde*I and *Pst*I (GibcoBRL, Life Technologies). The ~2200bp fragment contained the entire coding sequence for the fusion/insert. This was ligated into pVV16 (a gift from Dr. Varalakshmi Vissa, Colorado State University) and the resulting construct named, pIMP-Rv1086, was electroporated into competent *M. smegmatis* at 2.5 volts, 800 ohms, 25 µfarads. Cells were allowed to recover in LB broth for

90 min. and plated on LB agar with kanamycin (20 μg/ml). A single colony was chosen to start a liquid cultures in LB broth with kanamycin (20 μg/ml). The recombinant *M. smegmatis* strain containing vector pIMP-Rv1086 was grown to mid-log phase in LB broth with 20 μg/ml kanamycin. This genetic construct expressed the *M. tuberculosis* open reading frame Rv1086 with a C-terminal fusion to a chitin binding domain. Approximately 6 g (wet weight) of cells were harvested by centrifugation, washed with a 0.9% saline solution, and centrifuged again. The resulting pellet was resuspended in cell lysis and column buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA and 0.1% Triton X-100. The cells were disrupted by probe sonication on ice with a Sanyo Soniprep 150 (10 cycles of 60 s on and 90 s off), and the suspension was centrifuged at 20,000 X g for 20 min. The pellet was discarded and the supernatant loaded onto a 0.8 ml column of chitin beads (New England Biolabs) that had been equilibrated with the same buffer. The expressed protein was eluted from the column essentially as described by the manufacturer, except that the cleavage buffer contained 20 mM Tris-HCl (pH 8.0), 0.1% Triton, 150 mM NaCl, 30 mM dithiothreitol and 10% glycerol. The eluted fractions were assayed for Z-FPP synthase activity and the protein concentration was estimated after cold acetone precipitation using a BCA protein assay kit (Pierce).

C. Results

Purification of Z-FPP synthase- Z-FPP synthase was expressed in *M. smegmatis* as a fusion protein with a carboxy-terminal chitin binding domain. Affinity chromatography on a chitin column permitted a simple one-step protein purification (New England Biolabs). After the cells were harvested and disrupted, the 20,000 g supernate was applied to the chitin column and washed. Subsequent elution with a dithiothreitol containing buffer cleaved the Z-FPP synthase from the chitin binding domain via an

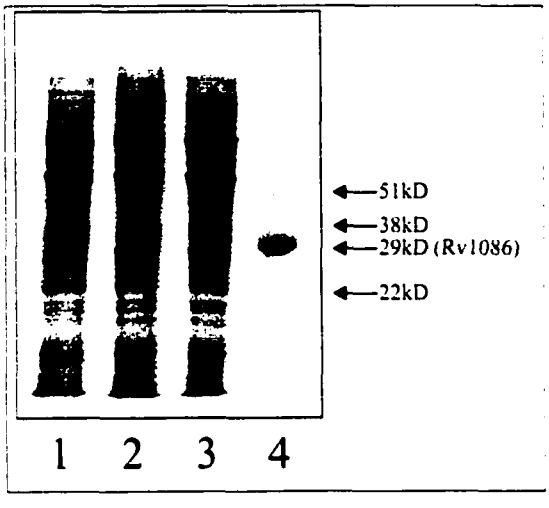


Figure 7.1. *SDS-PAGE analysis showing purification of Rv1086. Lane 1*: wildtype *M. smegmatis* cell lysate. *Lane 2*: Recombinant *M. smegmatis* cell lysate. *Lane 3*: Recombinant *M. smegmatis* cell lysate after application to chitin column. *Lane 4*: Eluate from column after dithiothreitol induced cleavage of intein site. The 12.5% polyacrylamide gel was stained with Coomassie Brilliant Blue R250. The calculated molecular weight of the Z-FPP synthase is 29.4 kD.

internal intein site. The column eluate was collected and subjected to SDS-PAGE analysis (Figure 7.1). The eluate was also assayed for [^{14}C]IPP incorporation into butanol extractable material with five different allylic primers, DMAPP (C_5), ω,E-GPP (C_{10}), ω,Z-neryl diphosphate (ω,Z-NPP; C_{10}), ω,E,E-FPP (C_{15}), and ω,E,E,E-GGPP (C_{20}). ω,E-GPP and ω,Z-NPP were the only functional substrates (data not shown). Assays with the other allylic primers did not produce any detectable radioactive product. [^{14}C]IPP incorporation into butanol extractable material in assays primed with ω,E-GPP or ω,Z-NPP was linear for at least forty min (data not shown). The product of the ω,E-GPP assay was analyzed for chain length and stereochemistry by TLC (Figure 7.2), confirming that the protein fraction shown in Figure 7.1 (Lane 4) synthesized ω,E,Z-FPP. The product of the ω,Z-NPP assay was also analyzed by TLC, demonstrating the enzyme's ability to synthesize a C_{15} molecule, presumably ω,Z,Z-FPP (data not shown).

Z-FPP Synthase Reaction Requirements- Z-FPP synthase was absolutely dependent on the presence of divalent cation for activity, addition of EDTA abolished the enzymatic activity (Table 7.1). Z-FPP synthase activity was supported by the addition of $MgCl_2$ or $MnCl_2$. The optimal concentration of $MgCl_2$ and $MnCl_2$ fell between 0.01 mM and 1.0 mM. Higher concentrations of $MgCl_2$ (between 1.0 mM-5.0 mM) reduced (with respect to optimal concentration) the activity, while high concentrations of $MnCl_2$

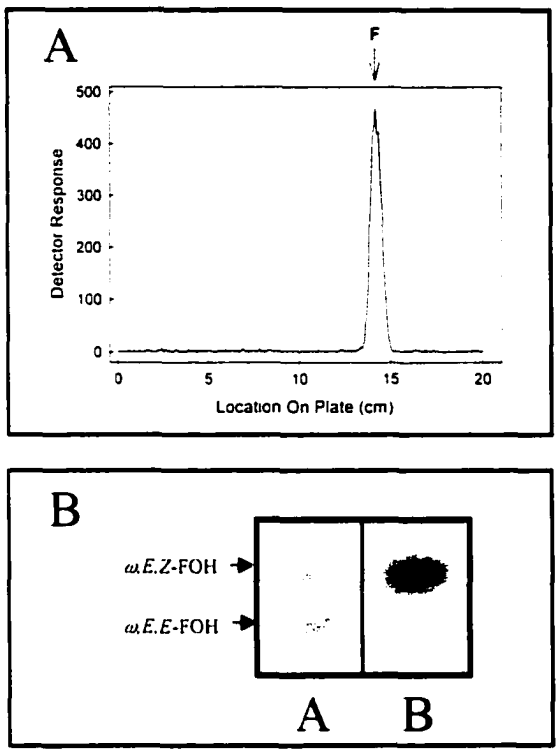


Figure 7.2. *Chain Length and stereochemical analysis of [^{14}C]IPP radiolabelled product synthesized by Z-FPP synthase primed with ω,E-GPP.* Isoprenyl diphosphate synthase activity was assayed as described in Chapter 4. The extracted isoprenyl diphosphates were dephosphorylated with potato acid phosphatase and spotted on reverse phase TLC plates in order to determine chain length. 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 (Bioscan Inc.). Standard polyprenols were located with anisaldehyde spray reagent. Migration of the non radioactive standard (F, Farnesol) is indicated with an arrow at the top of the Panel A. The material corresponding to farnesol was scraped from the reverse-phase TLC plate and extracted as described previously. The recovered farnesol was spotted onto a silica gel TLC plate and developed in toluene:ethyl acetate (7:3, v/v). Mixed isomers of farnesol (*ω,E,E*-farnesol and *ω,E,Z*-farnesol) along with the product of the Rv1086 assay primed with geranyl diphosphate were loaded onto a silica gel 60 TLC plate (Panel B, Lane A). Panel B, Lane B shows an autoradiogram of Lane A (exposure 24 hours).

Table 7.1- *Effect of Divalent Cations on Z-FPP synthase activity.* The enzyme preparation was preincubated with Bio-Rex 70 minus 400 mesh (sodium form, Bio-Rad) on ice for 20 min. *Z*-FPP synthase activity was tested for dependence on divalent cations in mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 2.5 mM dithiothreitol, 0.3% Triton X-100, 100 μM geranyl diphosphate, 30 μM [^{14}C]isopentenyl diphosphate and 60 μg protein in a final volume of 50 μl. EDTA, $MgCl_2$ or $MnCl_2$ was added to the assay mixtures at the indicated concentrations. Reactions were incubated for 10 min, stopped by the addition of water saturated with NaCl and extracted with *n*-butanol saturated with water.

	Divalent Cation	
Concentration	$MgCl_2$	$MnCl_2$
mM	Activity (pmol/mg/min)	
0	219	219
0.01	352	699
0.1	664	1409
1.0	704	872
5.0	326	20
EDTA (10 mM)	0	0

(1.0 mM and 5.0 mM) strongly inhibited it. The enzyme was not stimulated by $ZnCl_2$ and $CaCl_2$ at the concentrations tested (0.01 mM - 5 mM). *Z*-FPP synthase was also tested for optimal activity over a range of pH (5.5-9.5 in 0.5 increments). The enzyme had a broad peak of activity over pH 7- 8 (data not shown). When the rate of *Z*-FPP synthesis was measured in the presence of saturating ω,E-GPP and varying concentrations of [^{14}C]IPP (Figure 7.3A), a K_m value of 124 μM was calculated for the isoprenyl donor by nonlinear regression (Table 7.2). When the concentration of IPP was fixed and the concentration of ω,E-GPP or ω,Z-NPP was varied Michaelis constants of 38 μM and 16 μM were calculated (Figure 7.3 and Table 7.2).

Table 7.2. *Kinetic Constants of Z-FPP synthase.* Experimental conditions were as described for Figure 3. The data was subjected to nonlinear regression analysis using SigmaPlot for Windows version 4.01 (SPSS Inc.).

	Substrate		
	IPP	GPP	NPP
K_m (μM)	124	38	16
V_{max} (pmol/mg/min)	4328	2827	1098
Coefficient of Determination	0.99	0.99	0.99

Inhibition of Z-FPP Synthase- Compounds **1-4** (Scheme 7.1) and citronellyl diphosphate (Cit-PP, Figure 7.4A) were tested for the ability to inhibit the synthesis of *ω,E,Z*-farnesyl diphosphate by the *Z*-FPP synthase from *M. tuberculosis* and the synthesis of *ω,E,E*-FPP by the *E*-FPP synthase from pig brain (*Sus scrofa*). *Z*-FPP synthase was inhibited by compound **4** and Cit-PP (Figure 7.4B). The respective IC_{50} values were estimated to be 300 μM and 350 μM in the presence of 100 μM GPP. *E*-FPP synthase was inhibited only by Cit-PP with an IC_{50} of 125 μM under similar assay conditions(Figure 7.4C). Compounds **1**, **2** and **3** had no effect on either of the FPP synthases at the concentrations tested (data not shown).

D. Discussion

Little is known about *Z*-isoprenyl diphosphate synthases. Some early partial purification and characterization work has been published for bacterial[28;29] and eukaryotic[30-34] *Z*-isoprenyl diphosphate synthases, but it was not until Shimizu *et al.* purified undecaprenyl diphosphate synthase from *Micrococcus luteus* in 1998 that a gene and protein sequence were correlated with the biochemical data[35]. The complete sequencing of the *M. tuberculosis* H37Rv genome (and other genomes), has since revealed a wealth of information. Apfel *et al.* was able to find 28 homologs of *M. luteus* undecaprenyl diphosphate synthase using comparative genome queries[36]. *M. tuberculosis* is unique among organisms that have had their genomes sequenced in that there are

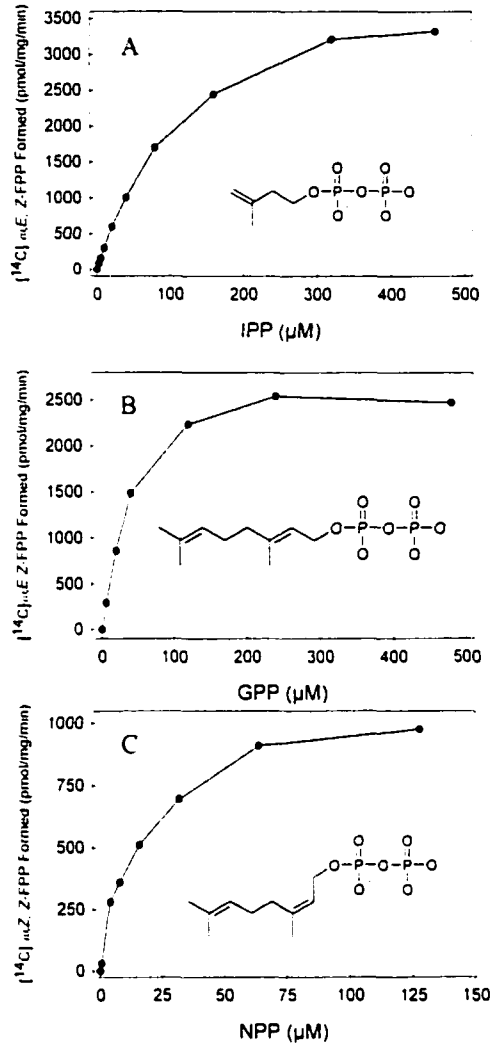


Figure 7.3. *The effect of substrate concentration on the rate of FPP synthesis by Z-FPP synthase.* Assays contained 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.3% Triton X-100 and 60 μg protein in a final volume of 50 μl. The assay results shown in Panel A contained 100 μM ω,*E*-geranyl diphosphate and increasing amounts [^{14}C]isopentenyl diphosphate (structure inset). The assays for Panel B contained 30 μM isopentenyl diphosphate and increasing amounts of ω,*E*-geranyl diphosphate (structure inset). The assays for Panel C contained 30 μM isopentenyl diphosphate and increasing amounts of ω,*Z*-neryl diphosphate (structure inset). Following incubation at 37°C for 10 min, the reactions were stopped with 1 ml water saturated with NaCl, and extracted with 1 ml butanol saturated with water. An aliquot was taken for liquid scintillation spectrometry.

two genes with homology to Z-isoprenyl diphosphate synthases (Rv1086 and Rv2361c), as opposed to the single Z-isoprenyl diphosphate synthase homolog typically found in the genomes of other organisms[36]. We previously identified the function of the enzymes encoded by Rv1086 (Z-FPP synthase) and Rv2361c (Z-decaprenyl diphosphate synthase)[9]. In this study, we report the purification, characterization and inhibition of Rv1086.

All isoprenyl diphosphate synthases (*E* and *Z*) studied thus far have a strict requirement for a divalent cation. However, the type of cation and the concentration required for optimal activity is unique to each enzyme. X-ray crystallography of avian *E*-FPP synthase, revealed that Mg^{2+} cations were positioned within the active site of the enzyme (bound to aspartate residues) while complexed with the diphosphate moiety of the substrate[38]. Undecaprenyl diphosphate synthase from *Lactobacillus plantarum* and *Escherichia coli* were both shown to require Mg^{2+} ions in order to bind IPP and a radiolabelled photolabile analogue of ω,E,E-FPP[37]. The mycobacterial *Z*-FPP synthase is no exception as it is stimulated by both Mg^{2+} and Mn^{2+}. It is likely that the divalent cation is required for substrate binding in a similar mechanism to that seen in *E*-isoprenyl diphosphate synthases.

Studies have shown that many *E*-FPP synthases can accept both DMAPP and ω,E-GPP as allylic substrates[38-42]. When synthesizing

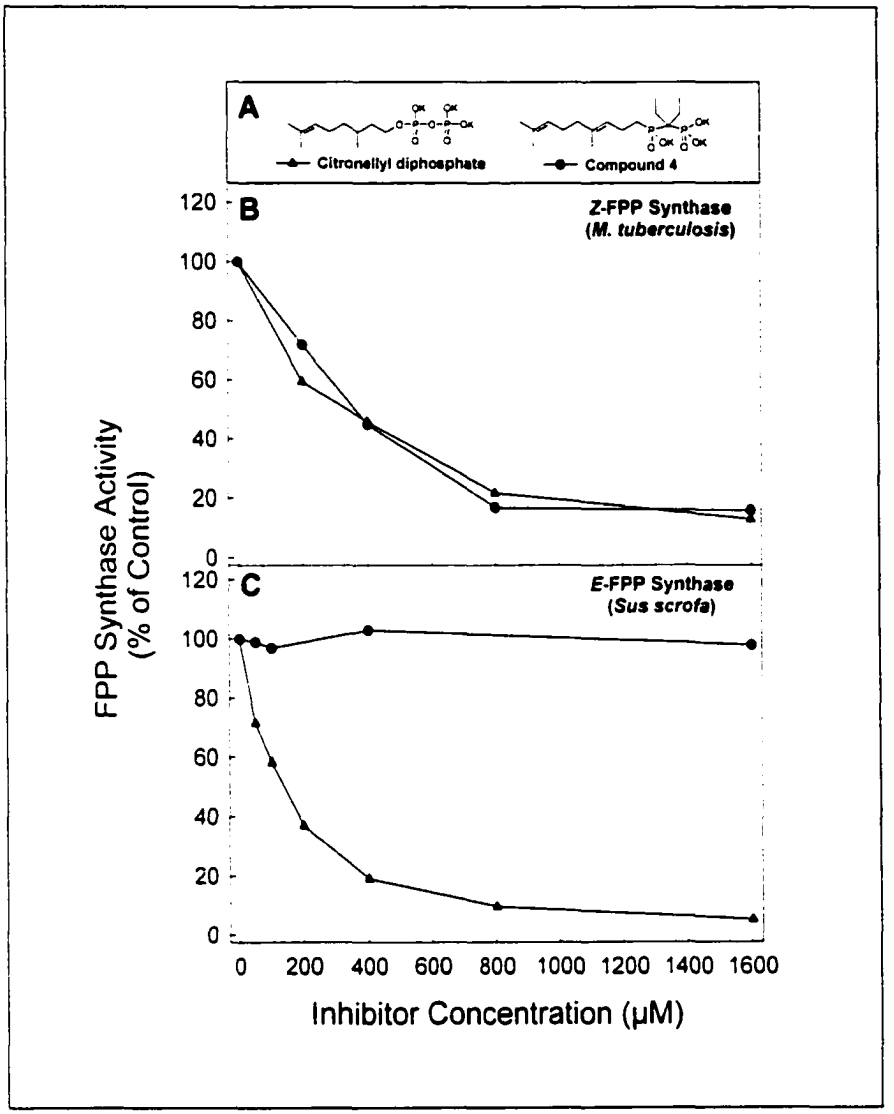


Figure 7.4. *Inhibition of Z-FPP Synthase and E-FPP Synthase with Geranyl Diphosphate Analogs.* Assay mixtures contained a buffer (Tris-HCl, MES and acetic acid pH 7.0), 10 mM sodium orthovanadate, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.3% Triton X-100 and 100 μM *ω,E*-GPP in a final volume of 50 μl. Assays for Panel B contained 60 μg of pure Rv1086 protein. Assays for Panel C contained 60 μg of cytosolic protein prepared from pig brain (*Sus scrofa*). Increasing concentrations of each inhibitor, citronellyl diphosphate (triangles) and compound **4** (circles) were added and the reactions were allowed to incubate for 20 min at 37°C. The products were extracted with *n*-butanol saturated with water and an aliquot was taken for liquid scintillation spectrometry.

ω,E,E-FPP from DMAPP, the enzyme completes two condensation reactions with IPP, releasing only trace amounts of the intermediate *ω,E*-GPP[3]. In contrast, DMAPP was not a functional substrate for the *Z*-FPP synthase. *ω,E*-GPP and *ω,Z*-NPP were the only effective allylic substrates tested, supporting the synthesis of *ω,E,Z*-FPP and *ω,Z,Z*-FPP, respectively. While little is known about the intracellular concentrations of IPP or the allylic substrates *ω,E*-GPP or *ω,Z*-NPP in *M. tuberculosis*, the observed Michaelis constants for these substrates are IPP, 124 μM; GPP, 38 μM; NPP, 16 μM. Long chain *Z*-isoprenyl diphosphate synthases have been shown to be capable of utilizing allylic primers with different stereochemistries as substrates[3,43]. If the Pol-P from *M. tuberculosis* is structurally similar to the Pol-P from *M. smegmatis* (*ω,E,polyZ*-decaprenyl phosphate), *ω,E*-GPP is the natural substrate of *Z*-FPP synthase. It is possible that *ω,Z*-NPP is a precursor to an, as yet, undescribed isoprenoid molecule in *M. tuberculosis*. Nevertheless, the *Z*-FPP synthase is a monofunctional enzyme and a separate enzyme must exist in *M. tuberculosis* that synthesizes either *ω,E*-GPP or *ω,Z*-NPP from DMAPP and IPP.

A comparison of the mycobacterial *Z*-FPP synthase with the *E*-FPP synthase from *Sus scrofa* brain was of interest because the two enzymes catalyze the addition of IPP to *ω,E*-GPP, but release products with opposite stereochemistry at the newly formed double

bond. Substrate analogs such as Cit-PP have been previously shown to inhibit pig liver *E*-FPP synthase[44;45]. Cit-PP and other substrate analogs are thought to bind the enzyme active site through non-specific lipophilic forces and a diphosphate binding force[44]. The enzyme activity is inhibited because Cit-PP lacks the allylic double bond in the 2 position (Figure 7.4A) and is not able to undergo the typical electrophilic alkylation reaction. As shown in Figure 7.4B and 7.4C, Cit-PP was effective at inhibiting both the *E*- and the *Z*-FPP synthases.

Compounds **1-4** (Scheme 7.1) were designed as analogs of ω,*E*-GPP in which the biologically labile diphosphate moiety was replaced by moieties that can act as stable isosters that possess different conformational and stereoelectronic characteristics, such as an unsubstituted phosphonoacetamidoxy (compound **1**), an α-ethyl substituted phosphonoacetamidoxy (compound **2**), a phosphonoacetamido group (compound **3**), or an α,α'-diethyl substituted [methylen(hydroxy)phosphoryl]methanphosphonic moiety (compound **4**). These isosteric moieties were chosen bearing in mind previous studies on isosters of the diphosphate group of prenyl diphosphates as squalene synthase[26], protein:farnesyl transferase[46], and protein:geranylgeranyl transferase inhibitors[47;48]. Compounds **1-3** had no effect on either the *Z*-FPP synthase or the *E*-FPP synthase (data not shown). Compound **4** specifically inhibited the *Z*-FPP synthase (Figure 7.4B), but had no effect on the activity of the *E*-FPP synthase (Figure 7.4C).

Despite its relatively high IC_{50}, the α,α'-diethyl substituted [methylen(hydroxy)phosphoryl]methanphosphonic moiety of compound **4** seems to possess the correct conformational and stereoelectronic features to selectively interact with the active site of the mycobacterial *Z*-FPP synthase, suggesting that the study of the active site of this previously uncharacterized enzyme could lead to the development of novel chemotherapeutic agents for the treatment of multiple drug resistant tuberculosis.

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Chapter 8.

ω,E,E-Farnesyl Diphosphate Synthase in *Mycobacterium tuberculosis*: An Atypical Bacterial Isoprenyl Diphosphate Synthase.

Open reading frame Rv3398c from the *M. tuberculosis* H37Rv genome[1] was identified as a homolog of known *E*-isoprenyl diphosphate synthases (Chapter 5). The chain length determining region of Rv3398c contains the FARM, DDXXD, and a single aromatic amino acid -5 to the FARM. This motif categorized the enzyme as a GGPP synthase type I[2]. In Wang and Ohnuma's model, the GGPP synthase type I represents the ancestral isoprenyl diphosphate synthase. Wang and Ohnuma's model also maintains that all isoprenyl diphosphate synthases with the GGPP synthase type I motif are found in archaebacteria. Further, all prokaryotic isoprenyl diphosphate synthases contain a 2 amino acid insertion in the FARM motif, DDX**XX**XD. Rv3398c was cloned into *M. smegmatis* and the recombinant protein fraction was analyzed for increased isoprenyl diphosphate synthase activity (compared to wildtype). The results have been prepared for publication in the Journal of Biological Chemistry.

A. Introduction

Isoprenyl diphosphate synthases catalyze the addition of isopentenyl diphosphate (IPP) to an allylic diphosphate

generating a longer allylic diphosphate[3,4]. Once the prenyl diphosphate has reached a physiologically relevant length it can undergo chain modification (dephosphorylation, condensation, cyclization) to create one of the 23,000 structurally and functionally diverse isoprenoid species. The enzymes responsible for chain elongation, isoprenyl diphosphate synthase, can be divided into two groups depending on whether they catalyze the formation of products with *E*-stereochemistry or of products with *Z*-stereochemistry. The *E*- and *Z*- isoprenyl diphosphate synthases do not share amino acid similarity[5,6].

To date, amino acid sequence alignments have been generated containing over fifty *E*-isoprenyl diphosphate synthases from diverse sources[2,4,7]. These studies have identified 5 regions of amino acid conservation, including two aspartate rich motifs, $DD(XX)_{1-2}D$. X-ray crystallography of the avian farnesyl diphosphate (FPP) synthase has shown that the aspartate motifs are necessary for binding the diphosphate moieties of the substrates[8,9]. The avian FPP synthase contains a large barrel-like cleft, made from 12 anti-parallel alpha helices. The aspartate residues line the walls of the barrel-like cleft and provide binding sites for the substrates. Site-directed mutagenesis experiments have defined key amino acids around the first aspartate rich motif (FARM) capable of directing the chain length of the product by changing the shape of the barrel-like cleft[10-13]. These amino acids serve as the basis for classifying *E*-isoprenyl diphosphate synthases[2].

In a recent review, Wang and Ohnuma used the chain length determining (CLD) region and the FARM signature motif to categorize *E*-isoprenyl diphosphate synthases[2]. The enzyme categories maintained the correct enzyme:product relationship for all isoprenyl diphosphate synthases in their model.

In this model, the farnesyl diphosphate synthases were divided into two classes, type I (eukaryotic) and type II (prokaryotic). Both of these classes presumably evolved from an enzyme which is similar to the archaebacterial geranylgeranyl diphosphate synthase type I (Figure 8.1). The GGPP synthase type I contains 1) the FARM motif DDXXD, and 2) a single aromatic amino acid in the fifth position before the FARM. The aromatic amino acid has been shown to regulate the chain length of GGPP synthases type I.

The FPP synthase type I is similar to the GGPP synthase type I in that it also contains the aspartate motif DDXXD, but in contrast, the FPP synthase type I contains two aromatic amino acids at positions -4 and -5 before the FARM. This change reduced the depth of the barrel-like cavity, converting the enzymatic activity from C_{20} to a C_{15} synthase.

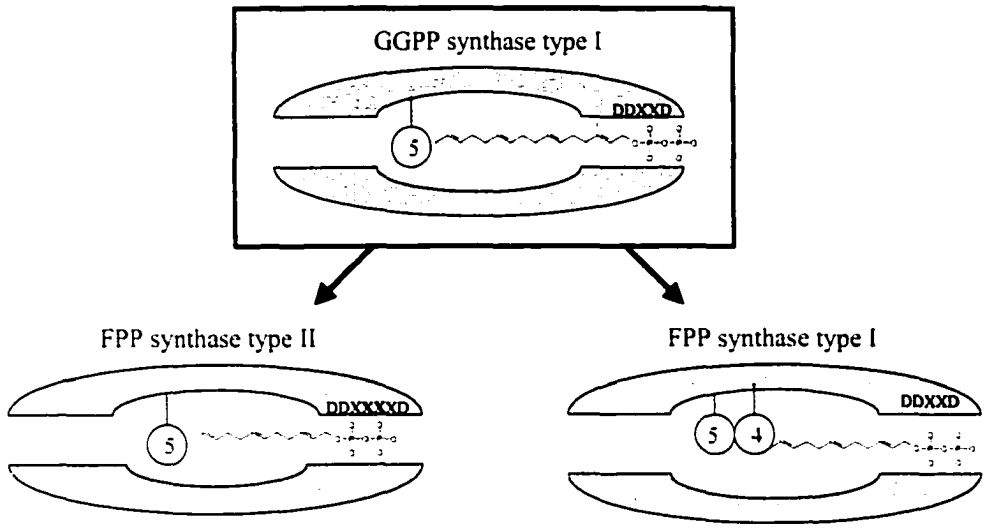

Figure 8.1- The evolution of FPP synthases from the ancestral GGPP synthase type I. The FPP synthase type I acquired an aromatic amino acid in the fourth position before the first aspartate motif, DDXXD. The FPP synthase type II acquired a two amino acid insertion in the first aspartate motif (DDX**XX**XD) and some additional uncharacterized modifications.

Type II *E*-farnesyl diphosphate synthases contain 1) the FARM DDX**XX**XD, and 2) a single aromatic amino acid in the -5 before the FARM. The characteristic change in the conversion of GGPP synthase type I to a FPP synthase type II was a two amino acid insertion within the FARM motif. However, mutagenesis studies have shown that this insertion is not enough to confer C_{15} synthetic activity. Therefore, additional changes must have occurred which have not been characterized.

We report that the *M. tuberculosis* open reading frame Rv3398c encodes a FPP synthase. This enzyme represents a new class of isoprenyl diphosphate synthase. It is the only prokaryotic isoprenyl diphosphate synthase that does not contain a two amino acid insertion in the FARM. The physiological functions for the FPP synthase in *M. tuberculosis* are discussed.

B. Experimental Procedures

General materials and methods were reviewed in Chapter 4. Those procedures relevant to this chapter are: subcellular fractionation, *in vitro* isoprenyl diphosphate synthase assays, enzymatic treatment of reaction products, analysis of radiolabelled product chain length, analysis of radiolabelled product stereochemistry, amino acid sequence analysis. The Chapter 8 specific cloning procedure is described below.

Cloning of Isoprenyl Diphosphate Synthase- Open reading frame Rv3398c in the *M. tuberculosis* H37Rv genome was identified as a potential isoprenyl diphosphate synthases based on its homology to known *E*-isoprenyl diphosphate synthases. Advanced BLAST searches were performed online at the National Center for Biotechnology Information. The following primers were designed to amplify open reading frame Rv3398c from H37Rv genomic DNA: 5'-GCGGTACAGACGAAAAGTACGGACTGC-3' and 5'-ACGCCTGTTC*GAATTC*GGTCATGC-3' An EcoRI restriction endonuclease site (underlined) was engineered into the N-terminal primer. PCR was performed on a

Perkin Elmer GeneAmp 2400 PCR System using 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 electroporated into *E. coli* compentent cells (XL-1 Blue). Cells containing plasmid were selected on LB agar containing kanamycin at a concentration of 20 μg/ml. Purified plasmids were subjected to restriction and sequence analysis. The resulting construct, named pMV-Rv3398c was electroporated into *M. smegmatis* mc^2155. In addition, pMV261 (without insert) was electroporated into *M. smegmatis* for use as a control. For electroporation, *M. smegmatis* cells were grown to late log phase in 500ml LB broth, washed seven times with ice-cold, sterile 10% glycerol and frozen at -70°C in 50 μl aliquots until used. Electroporation was performed at 2.5 volts, 800 ohms, 25 μfarads. Cells were recovered in LB broth for 90 min. and plated on LB agar with kanamycin (20 μg/ml). A single colony was chosen to start a liquid cultures in LB broth with kanamycin (20 μg/ml). Wildtype and recombinant (containing pMV621 empty vector or pMV261 with insert Rv3398c) *M. smegmatis* strains were grown to mid-log phase in LB broth (20 μg/ml kanamycin for recombinant strains).

C. Results

Recombinant M. smegmatis Overexpressing Rv3398c Increases [^{14}C]IPP Incorporation into Butanol-extractable Material - Rv3398c was cloned into the mycobacterial expression vector pMV261 and

expressed in *M. smegmatis*. Cytosolic protein from the wildtype and from the recombinant 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 protein preparation primed with DMAPP was 2-fold higher than the corresponding wildtype assay (data not shown). The Rv3398c recombinant protein also had 2-fold higher activity than the corresponding wildtype assay when primed with ω,E-GPP (data not shown).

TLC Analysis of the Products synthesized by the Rv3398c Overexpressor- The products of the above assays were enzymatically dephosphorylated to yield the corresponding prenyl alcohols, which were then applied to a reverse phase TLC plate for chain length analysis. When the products of the assays primed with DMAPP were compared (wildtype vs. recombinant), there was a small, but reproducible increase in the synthesis of farnesyl diphosphate and geranylgeranyl diphosphate by the recombinant protein (Figure 8.2). The assays primed with GPP showed a similar result (data not shown).

Assays Primed with both GPP and FPP Reveal that the Rv3398c is Overexpressing an FPP synthase- The data in Figure 8.2 suggests that the Rv3398c recombinant may be a GGPP synthase capable of releasing detectable amounts of intermediate, FPP. The

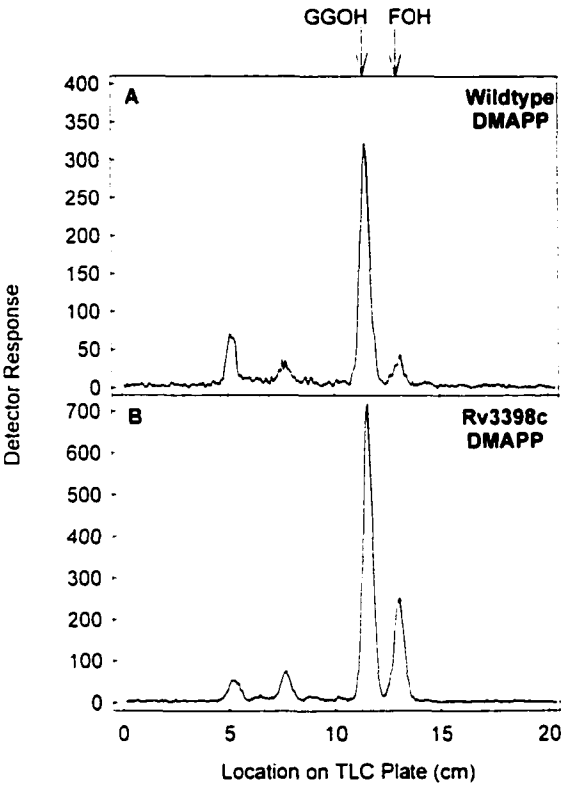

Figure 8.2- TLC analysis of [^{14}C]IPP radiolabelled products synthesized by cytosolic fractions from wildtype or recombinant *M. smegmatis*. Wildtype (Panel A) or recombinant expressing Rv3398c (Panel B) *M. smegmatis* cytosol was assayed in the presence of DMAPP. Isoprenyl diphosphate synthase activity was assayed and the extracted isoprenyl diphosphates were dephosphorylated with potato acid phosphatase as described in Chapter 4. Equal amounts of radioactivity were spotted on reverse-phase TLC plates. 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. Migration of non-radioactive standards (GGOH, Geranylgeraniol: FOH, Farnesol) are indicated with arrows at the top of the panels.

possibility remains that open reading frame Rv3398c encodes a FPP synthase and the background *M. smegmatis* GGPP synthase is depleting the product pool created by the overexpressed Rv3398c activity. To test this hypothesis, assays were conducted that used two allylic primers, GPP and FPP, in each assay mixture. This allowed the radiolabelled product, FPP, (produced by the addition of IPP to the allylic primer, GPP) to accumulate. The larger pool of cold FPP would reduce amount of radiolabelled FPP that is further elongated into GGPP by the background *M. smegmatis* enzymes. Wildtype protein, simultaneously primed with GPP and FPP, primarily produced GGPP (Figure 8.3A). Trace amounts of FPP and heptaprenyl diphosphate are also present. Figure 8.3B shows the assay of Rv3398c recombinant protein primed with GPP and FPP in which the primary product is FPP.

Stereochemical Analysis of the Double Primer Reaction Products Reveals that the FPP synthesized by Rv3398c Recombinant is in the ω,E,E-FPP stereoconfiguration- The FPP created by adding one molecule of [^{14}C]IPP to *ω,E*-GPP can have two possible stereochemistries, *ω,E,E*-FPP or *ω,E,Z*-FPP. Similarly the GGPP produced from the addition of one molecule of [^{14}C]IPP to *ω,E,E*-FPP can also have two possible stereochemistries, *ω,E,E,E*-GGPP or *ω,E,E,Z*-GGPP. It is possible to separate the stereoisomers by silica gel TLC. The radiolabelled products (FPP and GGPP) produced in the double primer assays that were dephosphorylated

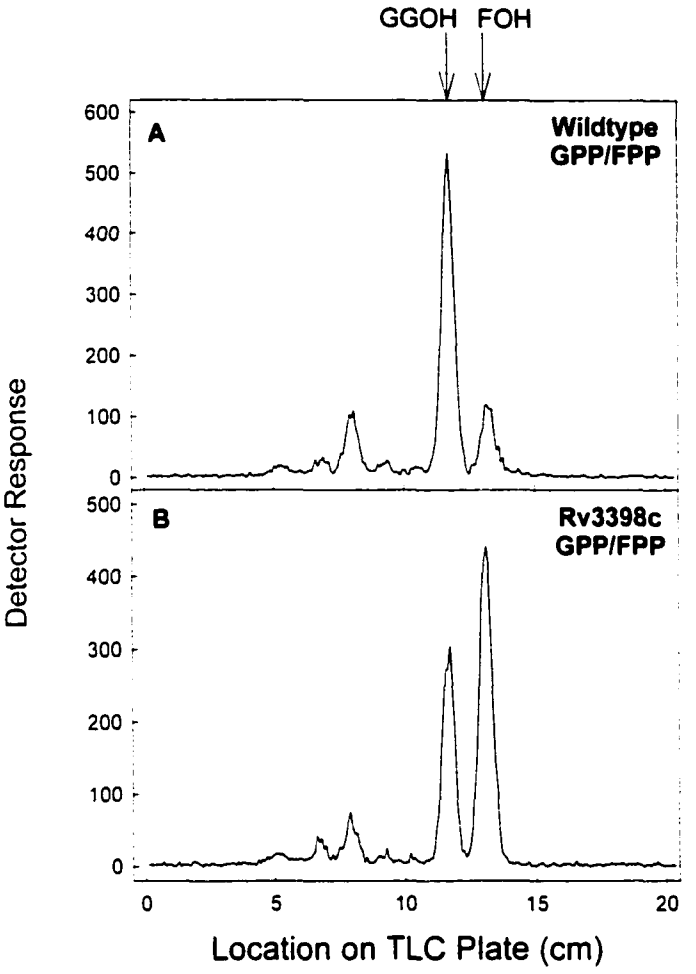

Figure 8.3- TLC analysis of [^{14}C]IPP radiolabelled products from assays primed with both GPP and FPP. Wildtype (Panel A) or recombinant expressing Rv3398c (Panel B) *M. smegmatis* cytosol was assayed in the presence of DMAPP and GPP. Isoprenyl diphosphate synthase activity was assayed and the extracted isoprenyl diphosphates were dephosphorylated with potato acid phosphatase as described in Chapter 4. Equal amounts of radioactivity were spotted on reverse-phase TLC plates. 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. Migration of non-radioactive standards (GGOH, Geranylgeraniol: FOH, Farnesol) are indicated with arrows at the top of the panels.

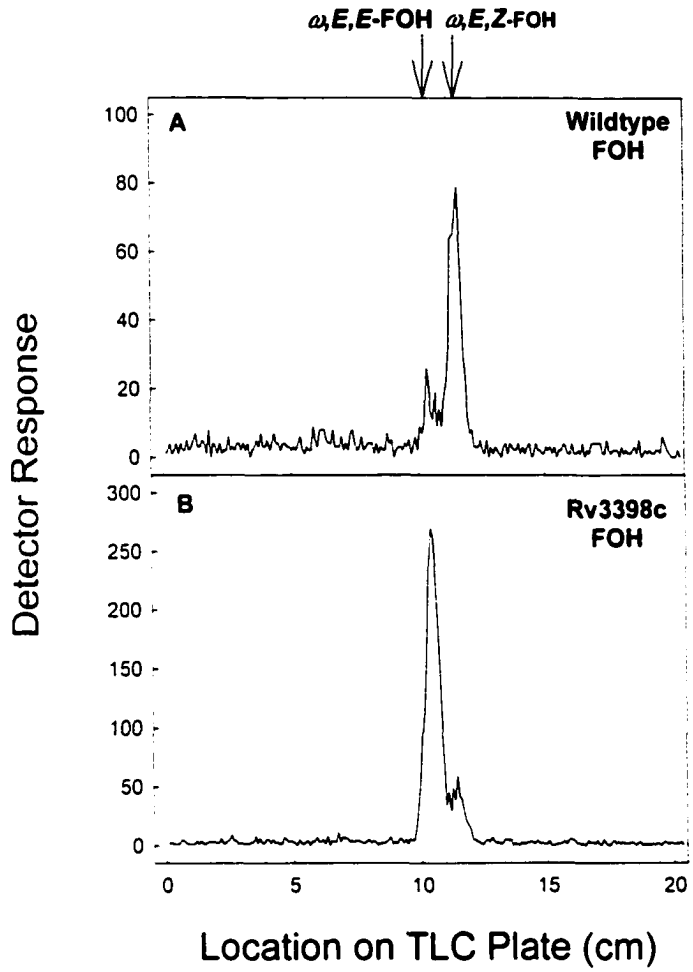

Figure 8.4- *Stereochemical analysis of farnesyl diphosphate enzymatically synthesized by the cytosol of wildtype (Panel A) and recombinant expressing Rv3398c (Panel B) M. smegmatis.* Assay conditions are described in Chapter 4. Assay products were dephosphorylated and subjected to reverse-phase TLC. The radiolabelled material corresponding to farnesol was scraped from the reverse-phase TLC plates and extracted as described in Chapter 4. The recovered farnesol was spotted onto a silica gel 60 TLC plate and developed in toluene:ethyl acetate (7:3, v/v). The location of the non-radioactive standards (mixed isomers of farnesol: *ω,E,E*-farnesol and *ω,E,Z*-farnesol) are shown above the panel.

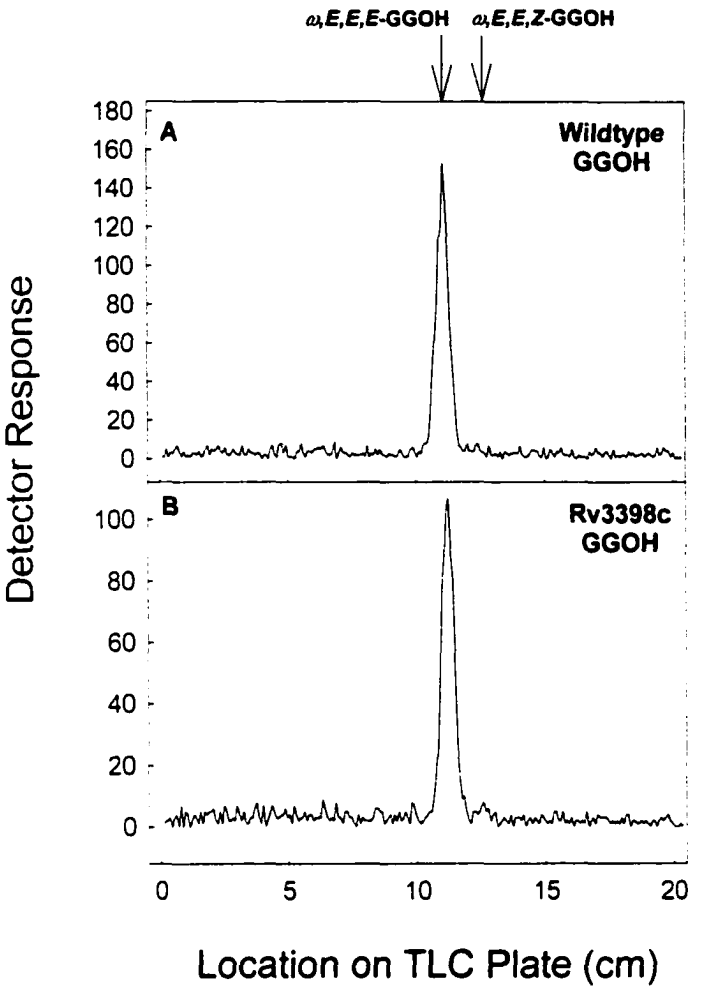

Figure 8.5- *Stereochemical analysis of geranylgeranyl diphosphate enzymatically synthesized by the cytosol of wildtype (Panel A) and recombinant expressing Rv3398c (Panel B) M. smegmatis.* Assay conditions are described in Chapter 4. Assay products were dephosphorylated and subjected to reverse-phase TLC. The radiolabelled material corresponding to geranylgeraniol was scraped from the reverse-phase TLC plates and extracted as described in Chapter 4. The recovered geranylgeraniol was spotted onto a silica gel 60 TLC plate and developed in toluene:ethyl acetate (7:3, v/v). The location of the non-radioactive standards (mixed isomers of geranylgeraniol: *ω,E,E,E*-geranylgeraniol and *ω,E,E,Z*-geranylgeraniol) are shown above the panel.

and separated by reverse phase TLC were recovered (scraped and extracted from the reverse phase gel) and applied to silica gel-60 TLC plates (Figures 8.4 and 8.5). The majority of the farnesol recovered from the wildtype lane migrated along with cold standard ω,E,Z-FOH which runs ahead of cold standard ω,E,E-FOH (Figure 8.4A). In contrast, the majority of the farnesol recovered from the Rv3398c overexpressor lane migrated along with ω,E,E-FOH (Figure 8.4B). The GGOH from both the wildtype and Rv3398c overexpressor lanes was in the ω,E,E,E-stereoconfiguration (Figure 8.5A and 8.5B).

D. Discussion

Open reading frame Rv3398c from the *M. tuberculosis* H37Rv genome encodes an ω,E,E-FPP synthase. This enzyme is unique because it lacks the amino acid motifs responsible for chain length determination for all other FPP synthases, and therefore must contain separate amino acid sequence features that determine chain length selectivity.

The mycobacterial FPP synthase was aligned with 25 other FPP and GGPP synthases (5 archaebacteria, 10 prokaryotic and 10 eukaryotic) and subjected to CLUSTAL analysis (Figure 8.6). While the type I eukaryotic and type II prokaryotic FPP synthases separated into distinct branches, the mycobacterial FPP synthase remained on a branch of its own. BLAST analysis of the mycobacterial FPP synthase retrieves, 1) putative isoprenyl

diphosphate synthases from Streptomyces spp. and Mycobacterium spp., both without the signature prokaryotic amino acid insertion (DDX**XX**XD) and 2) GGPP synthases type I from Archaeabacteria.

The function of the FPP synthase in *M. tuberculosis* is unknown. In most prokaryotes, *ω,E,E*-FPP is the C_{15} intermediate for the synthesis of *ω,E,E,polyZ*-undecaprenyl phosphate[14,15]. However, *M. tuberculosis* does not make *ω,E,E,polyZ*-undecaprenyl phosphate, but rather makes *ω,E,polyZ*-decaprenyl phosphate[16] and the C_{15} intermediate for this biosynthetic pathway is *ω,E,Z*-FPP[17]. *ω,E,E*-FPP does not seem to be a substrate for the synthesis of the octaprenyl diphosphate synthase, as the enzymatic activity identified in *M. tuberculosis* membranes was stimulated by the allylic substrate *ω,E,E,E*-GGPP, not *ω,E,E*-FPP[18].

We investigated the genome of *M. tuberculosis* H37Rv[1] for clues that may help identify the function of the *ω,E,E*-FPP synthase. The open reading frame adjacent to the *ω,E,E*-FPP synthase, Rv3397c, encodes a protein with homology to squalene and phytoene synthases, the isoprenoid enzymes responsible for the head to head condensation of the allylic diphosphates, FPP and GGPP, respectively. This suggests that *ω,E,E*-FPP might be used for squalene or sterol synthesis.

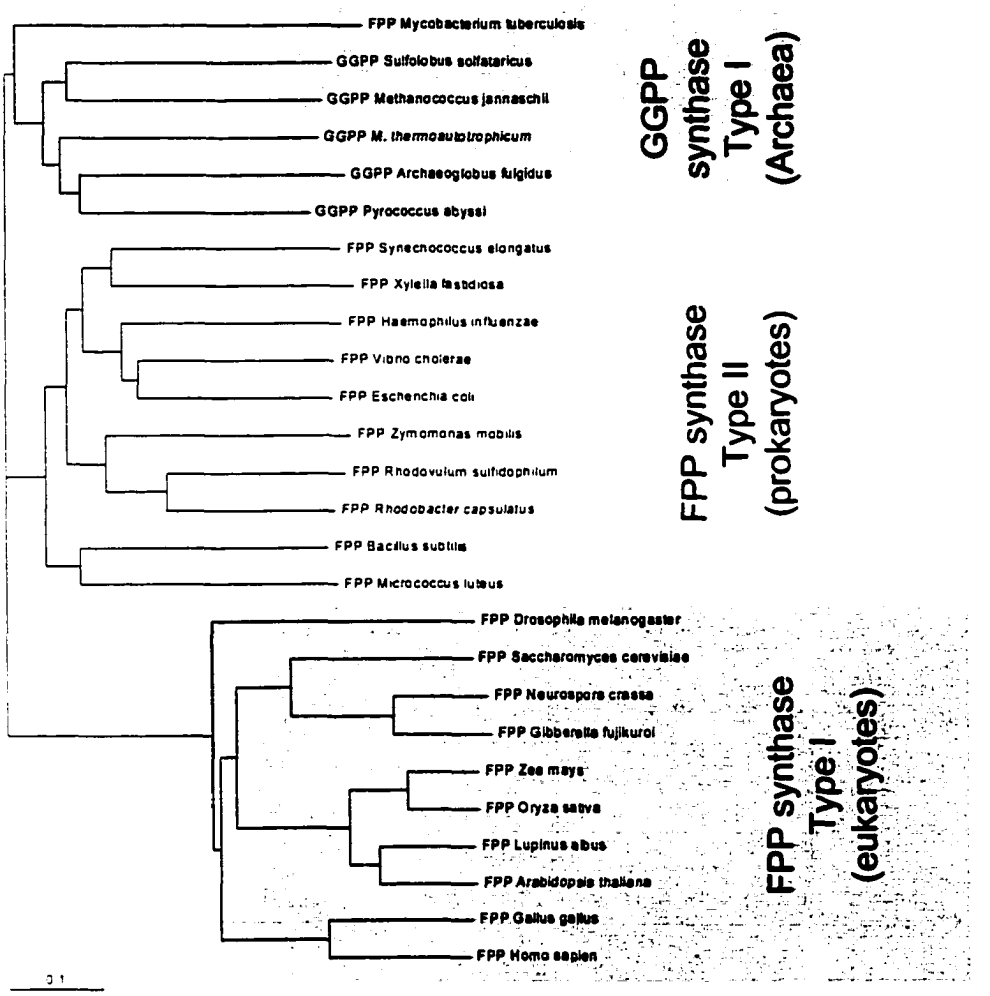

Figure 8.6- Clustal analysis of the mycobacterial FPP synthase with 25 FPP and GGPP synthases of archaebacterial, prokaryotic and eukaryotic origin: Accession numbers are in paranthesis: *Mycobacterium tuberculosis* (Q50727), *Drosophila melanogaster* (AAD27853), *Saccharomyces cerevisiae* (P08524), *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), *Pyrococcus abyssi* (C75139). Scale = branch distance for 0.1 nucleotide substitutions per site.

Recently, Lamb *et al.* reported that *M. smegmatis* synthesizes cholesterol[19]. *M. tuberculosis* has demonstrated the ability to uptake, modify and accumulate exogenous cholesterol[20]. Further, our laboratory has found that lipids extracted from *M. tuberculosis* are capable of binding the fluorescent polyene antibiotic, filipin, which selectively binds cholesterol and structurally related sterols (Crick, unpublished data). It is of considerable interest to further examine the possibility of that a cholesterol biosynthetic pathway exists in *M. tuberculosis*.

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Chapter 9

The Final Discussion

A. Summary

Isoprenoids are a diverse family of molecules based on a branched five-carbon isoprene unit[1,2]. Nature has assembled isoprene units into a vast array of compounds that play many different roles in cellular metabolism. However, the work in this dissertation has been primarily concerned with only polyprenyl phosphate molecules, or more specifically, *ω,E,polyZ*-decaprenyl phosphate from *M. tuberculosis*. Pol-P has been shown to be central to the synthesis of the prokaryotic cell wall[3,4]. The structurally unique mycobacterial cell wall is not an exception as Pol-P is involved in the synthesis of peptidoglycan, linker unit-arabinogalactan, lipomannan and lipoarabinomannan[5-7]. Pol-P may also be the terminal carrier in mycolic acid biosynthesis/attachment[8]. Despite its critical role in mycobacterial physiology, little is known about the synthesis of Pol-P.

To initiate the investigation of Pol-P, cell free extracts of *M. tuberculosis* were assayed for isoprenyl diphosphate synthase

activity. In the presence of cytosolic or membrane protein, allylic diphosphate primers were alkylated with [^{14}C]IPP. The products of the assays were extracted and analyzed for chain length and stereochemistry. From this data, it was apparent that *M. tuberculosis* contained several isoprenyl diphosphate synthase activities. The major products identified from the assays were: *ω,E,Z*-FPP, *ω,E,E,E*-GGPP, octaprenyl diphosphate and decaprenyl diphosphate. The data also suggested that *ω,E,Z*-FPP is the intermediate for decaprenyl diphosphate and that *ω,E,E,E*-GGPP is the intermediate for octaprenyl phosphate. When the information gathered from the *M. tuberculosis* assays was compared to what is known about Pol-P synthesis in *E. coli*[9] and *B. subtilis*[10-12], one major difference was apparent. *E. coli* and *B. subtilis* both contain *ω,E,E*-FPP which is the precursor to *ω,diE,polyZ*-undecaprenyl diphosphate, whereas *M. tuberculosis* contains *ω,E,Z*-FPP. The presence of *ω,E,Z*-FPP in *M. tuberculosis* accounts for the stereochemical difference of its final Pol-P molecule, (*ω,E,polyZ*-decaprenyl phosphate in *M. tuberculosis* vs. *ω,diE,polyZ*-undecaprenyl phosphate in *E. coli* and *B. subtilis*). Little is known about the interactions of Pol-P with the membrane and cell wall so it is not clear how one bacterium would benefit from the synthesis of one Pol-P molecule over the other.

The goal of this dissertation was to identify potential drug targets for the development of new chemotherapeutic agents

against tuberculosis. In order to accomplish this, it was necessary to identify proteins (and the corresponding genes) involved in the synthesis of *ω,E,polyZ*-decaprenyl phosphate. To begin, searches for isoprenyl diphosphate synthase homologs were conducted in the *M. tuberculosis* H37Rv genomic database[13]. Seven open reading frames were identified that had isoprenyl diphosphate synthase homology (Chapter 5). Rv3383c, Rv3398c and Rv2173 had homology to short chain *E*-isoprenyl diphosphate synthases. Rv0562 and Rv0989c had homology to medium chain *E*-isoprenyl diphosphate synthase and Rv2361c and Rv1086 had homology to *Z*-isoprenyl diphosphate synthases. All seven of the open reading frames were cloned into *M. smegmatis*. Protein from the recombinant *M. smegmatis* (expressing one of the seven open reading frames) and from wildtype *M. smegmatis* were assayed for isoprenyl diphosphate synthase activity. Three of the seven open reading frames were correlated with a change in isoprenyl diphosphate synthase activity. These results were presented in Chapters 6 and 8. One of the enzymes (Rv1086; Z-FPP synthase) was subject to purification, characterization and inhibition (Chapter 7). Expression of open reading frames Rv2173, Rv3383c, Rv0562 and Rv0989c did not confer increased isoprenyl diphosphate synthase activity. Data collected on each of the seven open reading frames is reviewed below.

Rv1086

Open reading frame Rv1086 of the *M. tuberculosis* H37Rv genome was identified by its homology to the *Z*-isoprenyl diphosphate synthase from *Micrococcus luteus*[14]. The enzyme's function was identified as a result of comparing wildtype *M. smegmatis* cell free extracts to the recombinant *M. smegmatis* cell free extracts. The increased isoprenyl diphosphate synthase activity was present in the cytosol and the membranes. Rv1086 catalyzes the addition of one molecule of IPP to ω,E-geranyl diphosphate. The product is ω,E,Z-farnesyl diphosphate. The *Z*-FPP synthase is the first short chain isoprenyl diphosphate synthase to catalyze a product with *Z*-stereochemistry. Previously, all short chain isoprenyl diphosphate synthases catalyzed products with *E*-stereochemistry. It was not possible to characterize the *Z*-FPP synthase in the over-expression assays, so it was purified using a novel expression vector. The *Z*-FPP synthase was only able to utilize the C_{10} substrates, ω,E-GPP and ω,Z-NPP. This provided indirect evidence that a C_{10}-isoprenyl diphosphate synthase exists in *M. tuberculosis*. However, a C_{10} intermediate has not been observed in any of the assays. *Z*-FPP synthase also showed an absolute requirement for a divalent cation, an optimal pH range from 7-8, a K_m of 124 μM for IPP, 38 μM for GPP and 16 μM for NPP. Inhibitors to the *Z*-farnesyl diphosphate synthase were designed and chemically synthesized as stable analogs of geranyl diphosphate in which the labile diphosphate moiety was replaced with stable moieties. Studies with these compounds revealed that

the active site of *Z*-farnesyl diphosphate synthase differs from *E*-farnesyl diphosphate synthase from pig brain *(Sus scrofa)*, a difference that may be significant enough to facilitate anti-tuberculosis drug development.

Rv2361c

Open reading frame Rv2361c was also identified by its homology to the *Z*-isoprenyl diphosphate synthase from *Micrococcus luteus*[14]. The comparative over-expression assays demonstrated that this enzyme is capable of adding seven isoprene units to a C_{15} substrate and releasing decaprenyl phosphate. If the product of Rv1086, *ω,E,Z*-farnesyl diphosphate, is the natural substrate of the decaprenyl diphosphate synthase then the stereochemistry of the isoprene units in decaprenyl diphosphate would be *ω,E,polyZ*, which is identical to the decaprenyl phosphate isolated from *M. smegmatis*[15]. However, our assays showed that the decaprenyl diphosphate synthase also utilized exogenously-supplied *ω,E,E*-FPP as a substrate. If the natural substrate of decaprenyl diphosphate synthase is *ω,E,E*-FPP, then the structure of decaprenyl diphosphate would be *ω,diE,polyZ*.

Rv3398c

Open reading frame Rv3398c was identified based on its homology to *E*-isoprenyl diphosphate synthases[16;17]. The comparative over-expression assays demonstrated that Rv3398c synthesizes *ω,E,E*-

farnesyl diphosphate. This is interesting because the primary structure of Rv3398c is different from the primary structures of other farnesyl diphosphate synthases. Rv3398c does not contain the type I (eukaryotic) nor the type II (prokaryotic) farnesyl diphosphate synthase signature motifs[17]. Instead, it has the same signature motif as the geranylgeranyl diphosphate synthase from *Archaea*. The mycobacterial FPP synthase represents a previously undescribed class of FPP synthases, which contains a novel chain length determining mechanism.

In contrast to Rv1086 and Rv2361c, the Rv3398c open reading frame appears to be part of an isoprenoid biosynthetic cluster in the genome of *M. tuberculosis*[13]. This cluster contains: Rv3377c which encodes a kaurene synthase homolog. Typically found in plants, a kaurene synthase catalyzes the cyclization of geranylgeranyl diphosphate to ent kaurene, an intermediate in gibberelin synthesis. Rv3379c encodes a deoxyxylulose 5-phosphate synthase (DXS) homolog. In fact, *M. tuberculosis* actually contains two DXS homologs (the other being Rv2682c). It is presently unclear which of these (or both in the case of a heterodimer) play a role in IPP synthesis. Rv3382c encodes a LytB homolog. LytB has recently been postulated to have a role in IPP synthesis[18]. Rv3383c encodes an isoprenyl diphosphate synthase homolog. This gene was subjected to the comparative over-expression assays, however no change in isoprenyl diphosphate synthase activity was observed. Rv3397c encodes a phytoene/squalene synthase homolog.

It looks as though Rv3397c shares a promoter with Rv3398c, therefore it could be a squalene synthase (which catalyzes the head to head condensation of two FPP molecules, a required step for sterol and hopanoid synthesis). Lastly, Rv3389c encodes an estradiol 17-Beta dehydrogenase homolog and Rv3409c encodes a cholesterol oxidase homolog.

Rv3383c

Open reading frame Rv3383c was identified as an *E*-isoprenyl diphosphate synthase homolog[16;17]. The comparative over-expression assays did not reveal any increased isoprenyl diphosphate synthase activity. Rv3383c still remains a candidate for an isoprenoid biosynthetic gene because it is part of the isoprenoid biosynthetic cluster discussed above.

Rv0562

Open reading frame Rv0562, an *E*-isoprenyl diphosphate synthase homolog[15], did not possess isoprenyl diphosphate synthase activity in our assays. As it had homology to the medium chain isoprenyl diphosphate synthase, it is likely that this protein requires another gene product for catalysis[12]. The genomic arrangement of the heptaprenyl diphosphate synthases component I and component II of *B. subtilis* could be helpful in identifying the subunit[19]. In *B. subtilis*, component I and component II are on opposite sides of *menH*, a gene encoding a methyltransferase involved in menaquinone synthesis. *M. tuberculosis* has a similar arrangement

of menaquinone biosynthetic genes. Rv0562 is two open reading frames away from a putative methyltransferase gene (annotated *ubiE*). Adjacent to *ubiE* is an open reading frame that encodes an unknown protein (Rv0559c). This gene is a good candidate to encode the heptaprenyl diphosphate synthase component I homolog.

Expression of open reading frames Rv2173 and Rv0989c was not correlated with a change in isoprenyl diphosphate synthase activity. Even though open reading Rv0989c has homology to *E*-isoprenyl diphosphate synthases, it lacks many key amino acids residues that have been shown to be essential for catalysis[20]. In hindsight, it is not surprising that Rv0989c lacked isoprenyl diphosphate synthase activity. On the other hand, Rv2173 has high homology to *E*-isoprenyl diphosphate synthases, and contains all of the essential amino acids.

B. Conclusion

The work described in this dissertation barely scratches the surface of isoprenoid metabolism in *M. tuberculosis*. However, the pathway for the synthesis of Pol-P from DMAPP and IPP is now reasonably well understood (Figure 9.1). First, there is an unidentified enzyme that catalyzes the formation of ω,E-GPP from DMAPP and IPP. Second, *Z*-FPP synthase, encoded by open reading frame Rv1086, utilizes ω,E-GPP as a substrate, adds one IPP molecule and releases ω,E,Z-FPP. Lastly, ω,E,Z-FPP is then the

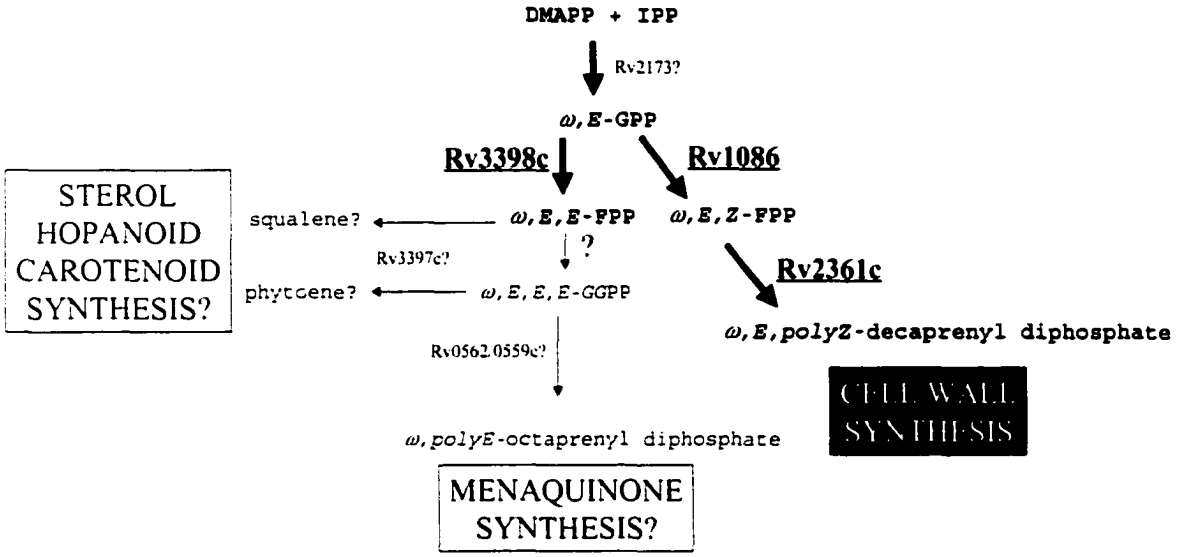

Figure 9.1- Isoprenoid synthesis in *M. tuberculosis*. DMAPP and IPP condense to form GPP. GPP is the allylic substrate for enzyme encoded by the open reading frame Rv1086, which produces *ω,E,Z*-FPP. *ω,E,Z*-FPP is the the substrate for the enzyme encoded by open reading frame Rv2361c, which releases decaprenyl diphosphate, the diphosphate precursor to the polyprenyl phosphate molecule involved in cell wall synthesis. GPP can also act as the allylic substrate of an *E*-isoprenyl diphosphate synthase (encoded by open reading frame Rv3398c) that synthesizes *ω,E,E*-FPP. This molecule could be an intermediate for the synthesis of *ω,E,E,E*-GGPP, but it could also be an intermediate for a squalene derived isoprenoid. *ω,E,E,E*-GGPP appears to be the substrate for an octaprenyl diphosphate synthase, which might be involved in menaquinone synthesis or for the synthesis of some unknown isoprenoid species. The *ω,E,E,E*-GGPP could also be used for carotenoid biosynthesis by way of a phytoene synthase. The Rv-numbers that are bold and underlined have been biochemically defined in this dissertation.

substrate for a decaprenyl diphosphate synthase, encoded by open reading frame Rv2361c, which adds seven more molecules of IPP. It is not known how decaprenyl diphosphate is converted to decaprenyl phosphate. Two enzymes have been identified in *Staphylococcus aureus* (reviewed in Rogers *et al.*[21]). The first catalyzes the dephosphorylation of undecaprenyl diphosphate to the free alcohol, undecaprenol. The second, can mono-phosphorylate the free alcohol to undecaprenyl phosphate. It is generally believed that this system regulated cell wall synthesis by controlling the amount of Pol-P present in the cell. However, other evidence suggests that this system may only exist in *S. aureus*.

Research over the last year has provided some insight that may help identify the suspected GPP synthase in *M. tuberculosis*. The GPP synthase from peppermint oil gland was recently cloned[22]. It came surprisingly that this enzyme is a heterodimer, with both subunits resembling *E*-isoprenyl diphosphate synthase. However, the smaller of the two subunits lost the FARM and SARM motifs. An investigation into the *M. tuberculosis* genome unfortunately revealed that *M. tuberculosis* does not contain homologs to the subunits of the GPP synthase. In a different study, Narita *et al.* succeeded in converting the *B. stearothermophilus* FPP synthase into a GPP synthase by adding an aromatic amino acid in position -4 to the FARM[23]. It is possible that this strategy occurred in nature too. Open reading frame Rv2173 from *M.*

tuberculosis, has homology to Rv3398c, the *ω,E,E*-FPP synthase. However in the chain length determining region, in place of the aromatic phenylalanine residue in position -5 to the FARM, there is a tryptophan residue. Perhaps this amino acid substitution shortened the active site and reduced the chain elongation capacity to a GPP synthase. Of course, this speculation is no substitute for biochemistry.

Many questions remain unanswered regarding *M. tuberculosis* isoprenoid biosynthesis. The function of octaprenyl diphosphate, identified in the *M. tuberculosis* cell free extracts is unknown. Also, the function of *ω,E,E*-FPP (product of Rv3398c) is not clear. It will be interesting to see if the adjacent gene, Rv3397c, encodes a functional squalene synthase. Perhaps these genes are involved in sterol or hopanoid synthesis. If the number and type of genes included in the *M. tuberculosis* isoprenoid biosynthetic cluster is any indication, many more isoprenoid structures will be found in *M. tuberculosis*.

It will be interesting to confirm the identification more isoprenyl diphosphate synthases that fail to conform to the evolutionary scheme of Wang and Ohnuma. *Streptomyces* spp. contains isoprenyl diphosphate synthase homologs that lack the two amino acid insertion in the FARM (similar to the mycobacterial homologs), however, no biochemistry has been published to show isoprenyl diphosphate synthase activity.

Quaternary structure analysis of the *E*-FPP synthase from *M. tuberculosis* may reveal a novel chain length determining mechanism. Photo-probe labeling, site-directed mutagenesis and quaternary structural analysis of the *Z*-FPP synthase will reveal which residues in the *Z*-isoprenyl diphosphate synthases participate in catalysis, substrate binding and chain elongation. Resolving the crystal structure will also allow a close comparison of the short chain *E*- and *Z*-FPP synthases, enzymes that bind identical substrates but release products with opposite stereochemistry. This comparative data may help in the rational design of new chemotherapeutic agents.

In closing, three enzymes were identified (*E*-FPP synthase, *Z*-FPP synthase and a decaprenyl diphosphate synthase) each of which are unique when compared to the known isoprenyl diphosphate synthases. It is necessary to determine the essentiality of these enzymes to the bacterium before they can be exploited for anti-tuberculosis drug design. Hopefully the knowledge gained from these experiments, both in the field of isoprenoid biochemistry and in the field of *M. tuberculosis* physiology, will encourage further investigation to answer the many questions that remain.

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