Functional Characterization and Evolution of the Isotuberculosinol Operon in Mycobacterium Tuberculosis and Related Mycobacteria

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Abstract
Terpenoid metabolites are important to the cellular function, structural integrity, and pathogenesis of the human-specific pathogen Mycobacterium tuberculosis (Mtb). Genetic and biochemical investigations have indicated a role for the diterpenoid isotuberculosinol (isoTb) early in the infection process. There are only two genes (Rv3377c and Rv3378c) required for production of isoTb, yet these are found in what appears to be a five-gene terpenoid/isoprenoid biosynthetic operon. Of the three remaining genes (Rv3379c, Rv3382c, and Rv3383c), previous work has indicated that Rv3379c is an inactive pseudo-gene. Here we demonstrate that Rv3382c and Rv3383c encode biochemically redundant machinery for isoprenoid metabolism, encoding a functional 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB) for isoprenoid precursor production and a geranylgeranyl diphosphate (GGPP) synthase, respectively, for which the Mtb genome contains other functional isozymes (Rv1110 and Rv0562, respectively). These results complete the characterization of the isoTb biosynthetic operon, as well as further elucidating isoprenoid metabolism in Mtb. In addition, we have investigated the evolutionary origin of this operon, revealing Mtb-specific conservation of the diterpene synthase genes responsible for isoTb biosynthesis, which supports our previously advanced hypothesis that isoTb acts as a human-specific pathogenic metabolite and is consistent with the human host specificity of Mtb. Intriguingly, our results revealed that many mycobacteria contain orthologs for both Rv3383c and Rv0562, suggesting a potentially important role for these functionally redundant GGPP synthases in the evolution of terpenoid/isoprenoid metabolism in the mycobacteria.

Keywords
isoprenoid biosynthesis, molecular evolution, terpenoids, virulence

Disciplines
Biochemistry, Biophysics, and Structural Biology | Ecology and Evolutionary Biology | Genetics and Genomics

Comments
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Functional characterization and evolution of the isotuberculosinol operon in Mycobacterium tuberculosis and related mycobacteria

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INTRODUCTION

Each year, Mycobacterium tuberculosis (Mtb) accounts for ~1.7 million deaths as the main causative agent of the human disease Tuberculosis (Rohde et al., 2007), and the ability of this deadly pathogen to infiltrate macrophage cells from the mammalian immune system remains an active area of investigation (Russell, 2007). While the complete pathogenic mechanisms remains unknown, the bacteria appear to launch a multi-factorial attack with both cell wall avoidance and arrest of the phagosome (Russell et al., 2010), with many bacterial genes involved in pathogenesis (Joshi et al., 2006). Notably, a mutational screen for genes involved in the early infection process highlighted a role for Rv3377c and Rv3378c, loss of which reduced the ability of Mtb to block acidification of the engulfing phagosome compartment, leading to suppressed bacterial proliferation in macrophage cell cultures (Peth et al., 2010). These genes have since been characterized as encoding a pair of sequentially acting diterpene synthases responsible for the production of isotuberculosinol (isoTb; Nakano et al., 2005a,b; Mann et al., 2009b; Maugel et al., 2010, Prach et al., 2010; Spangler et al., 2010; Hoshino et al., 2011; Mann et al., 2011b; Nakano et al., 2011), a diterpenoid capable of arresting phagosomal maturation in vitro (Mann et al., 2009b). Thus, isoTb appears to play an immunomodulatory role in the Mtb infection process (Mann and Peters, 2012).

Terpenoid metabolites are important to the cellular function, structural integrity, and pathogenesis of the human-specific pathogen Mycobacterium tuberculosis (Mtb). Genetic and biochemical investigations have indicated a role for the diterpenoid isotuberculosinol (isoTb) early in the infection process. In particular, only two genes (Rv3377c and Rv3378c) are required for production of isoTb, yet these are found in what appears to be a five-gene terpenoid/isoprenoid biosynthetic operon. Of the three remaining genes (Rv3379c, Rv3382c, and Rv3383c), previous work has indicated that Rv3379c is an inactive pseudo-gene. Here we demonstrate that Rv3382c and Rv3383c encode biochemically redundant machinery for isotuberculosinol metabolism, encoding a functional 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB) for isoprenoid precursor production and a geranyl/geranyl diphosphate (GGPP) synthase, respectively, for which the Mtb genome contains other functional isozymes (Rv1110 and Rv0662, respectively). These results complete the characterization of the isoTb biosynthetic operon, as well as further elucidating isoprenoid metabolism in Mtb. In addition, we have investigated the evolutionary origin of this operon, revealing Mtb-specific conservation of the diterpene synthase genes responsible for isoTb biosynthesis, which supports our previously advanced hypothesis that isoTb acts as a human-specific pathogenic metabolite and is consistent with the human host specificity of Mtb. Intriguingly, our results revealed that many mycobacteria contain orthologs for both Rv3382c and Rv0662, suggesting a potentially important role for these functionally redundant GGPP synthases in the evolution of terpenoid/isoprenoid metabolism in the mycobacteria.

Keywords: isoprenoid biosynthesis, molecular evolution, virulence, terpenoids
isoprenyl diphosphates such as the 10-carbon geranyl diphosphate (GPP), 15-carbon farnesyl diphosphate (FPP), and 20-carbon diterpenoid precursor geranylgeranyl diphosphate (GGPP). These are then used in cell wall assembly (e.g., the further elongated decaeryl diphosphate), synthesis of other essential biomolecules (e.g., menaquinone), as well as isoTb biosynthesis. In the isoTb operon, Rv3379c is homologous to the deoxy-xylulose-5-phosphate synthases that initiate the MEP pathway, and is annotated as dxs2, although it has been reported to be an inactive pseudo-gene (Bailey et al., 2002). Rv3382c encodes a putative 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, the LytB that catalyzes the final step of the MEP pathway, producing FPP and DMAPP (Rohdich et al., 2002), but this has not previously been investigated. Rv3383c encodes a member of the isoprenyl diphosphate synthase (IDS) family, which generally catalyze isoprenyl diphosphate chain elongation. Accordingly, the IDS encoded by Rv3383c is likely to produce the GPP required for isoTb biosynthesis, but this also has not yet been investigated. Here, we not only verify the biochemical function of both Rv3382c and Rv3383c, but also the presence of robust isozymes catalyzing the same reactions encoded elsewhere in the Mtb genome, suggesting functional redundancy consistent with the reported genetic screens. In addition, bioinformatics analysis of the available mycobacterial genome sequences indicates asymmetric conservation of isoTb biosynthetic machinery in Mtb relative to the greater Mycobacterium tuberculosis complex (MTC), consistent with a previously hypothesized role for isoTb in the highly infectious nature of Mtb in humans, along with the surprising finding of wider conservation of dual GGPP synthases in a sub-group of mycobacteria.

**MATERIALS AND METHODS**

**GENERAL**

All trans (E) isomers of GPP, FPP, and GGPP were purchased from Isoprenoids, IC (Tampa, FL, USA). Unless otherwise noted, all other chemicals were purchased from Fisher Scientific (St. Louis, MO, USA) and all molecular biology reagents were purchased from Invitrogen (Carlsbad, CA, USA). Gas chromatography-flame ionization (GC-FID) detection was carried out using an Agilent 6890 GC-FID (Agilent Technologies, Santa Clara, CA, USA) using a previously described method (Schmidt and Gershenzon, 2008).

**BIOINFORMATICS**

Genes of interest were identified via basic local sequence alignment (BLAST) against the *M. tuberculosis* H37Rv genome (Cole et al., 1998). Phylogenetic analysis of GGPP synthases (GGPPS) was accomplished via UPGMA modeling of amino acid sequences (Sneath and Sokal, 1973), using CLC Sequence Viewer 6.0 (CLC Bio, Cambridge, MA, USA). Complete operon analysis was performed against publicly available sequences of *Mtb* diversity strains (Hershberg et al., 2008), *M. canettii* (Hershberg et al., 2008), *M. marinum* (Stinear et al., 2008), and *M. ulcerans* (Stinear et al., 2007), as well as previously published sequences of *M. bovis* (Garnier et al., 2003), and *M. bovis* BCG (Brosch et al., 2007), and the pre-publication reads of *M. africanum* (available via the Sanger Institute). Genomic regions were aligned using BLAST analysis and synteny mapping via the Tuberculosis Database (Reddy et al., 2009).

**CLONING**

Genes of interest were cloned by PCR amplification from *Mtb* CDC1551 genomic DNA and confirmed via complete sequencing. These were transferred into pENTR/SD/D-TOPO (Gateway, Invitrogen) by directional recombination. Rv2173 and Rv0562 were modified to change the first codon to ATG to enable expression in *Escherichia coli*, for which purpose all genes were recombined into pDEST17 expression vectors for production with an N-terminal 6xHis tag.

**LytB COMPLEMENTATION**

LytB complementation was initially assessed by colony counting assays, carried out in three individual experiments, each in duplicate. 50 ng of plasmid (pDEST14) containing Rv3382c or Rv3383c were each electroporated into 100 μL electro-competent *E. coli* cells containing LytB under arabinose promoter control (McAteer et al., 2001) and were plated on NZY medium in the absence of arabinose, which allows only for growth of LytB complemented cells. Electroporated *E. coli* cells plated in the presence and absence of 0.2% arabinose were used as controls.

**FIGURE 1 | Proposed isoTb biosynthetic operon.** The five-gene operon including the two previously characterized diterpene synthases required for isoTb biosynthesis and the proposed roles of the remaining uncharacterized genes in the biosynthetic pathway. Genes depicted in the order suggested by their numbering rather than orientation of open reading frame.
More quantitative analysis of LytB activity was accomplished by comparison of relative growth rates, carried out in triplicate. Colonies from Plasmid (+) plates were grown to saturation in LB medium overnight. Cultures were diluted 1/100 into fresh LB medium containing 0.5 mM isopropylthiogalactoside (IPTG) and grown for 1 h, then diluted 1/50 into media containing 50 μg/ml carbenicillin and 0.2% (+v/v) glucose to inhibit the arabinose promoter and thus completely inhibit any expression of E. coli LytB. After 220 min, cultures were diluted 1/5 into medium containing 0.2% (+v/v) arabinose, thus activating the chromosomal LytB and allowing for comparative analysis of the relative health of both complemented strains (i.e., to demonstrate that expression of Rv3382c did not exert any toxicity relative to expression of Rv1110 instead). Culture growth was monitored every 20 min by measuring OD600.

**PUTATIVE GGPP SYNTHASE EXPRESSION**

Putative GGPP synthase genes from pDEST17 were transformed into the C41 strain of E. coli (Lucigen, Middleton, WI, USA), and plated on NZT agar with carbenicillin (50 μg/ml) selection. Colonies were inoculated in 50 ml of NZV medium and grown to an optical density at 600 nm (OD600) of 0.6 at 37°C for 16–18 h incubation. Cells were harvested via centrifugation and lysed in 1 mL lysis buffer (10 mM Tris-Cl, pH 6.8, 10% glycerol, 1 mM dithiothreitol) via sonication. These lysates were clarified via centrifugation and assayed for GGPP synthase activity. As E. coli does not harbor contaminating GGPP synthases (Burke and Crotteau, 2002), any resulting production of GGPP was attributed to expression of the recombinant gene.

**GGPP SYNTHASE ASSAY**

Assay buffer (50 mM sodium phosphate (pH 7.0), 10% glycerol, 0.2% (+v/v) glucose to inhibit the arabinose promoter and thus completely inhibit any expression of E. coli LytB) was added to clarified lysates prepared as described above. This assayed mixture was then incubated at 37°C for 24 h to allow for conversion of IPP to GGPP. The reaction was halted via incubation at 100°C for 5–10 min. After cooling, 1.5 U calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, MA, USA) was added according to manufacturer protocol. The phosphatase reaction was incubated overnight at 37°C and extracted thrice with 1 mL hexanes. Samples were concentrated and analyzed via GC-FID, and compared to similarly, dephosphorylated authentic GGPP.

**KINETIC ANALYSIS OF Rv3383c AND Rv0562**

The 6x-His tagged versions of these enzymes were purified via Ni-NTA Superflow resin (Novagen, Merck, Germany) following the manufacturer directions. Protein was analyzed via denaturing gel electrophoresis (SDS-PAGE), demonstrating >90% purity. Enzymes were assayed as described above, except using a substrate gradient of IPP (0.001–10 μM). Rates of IPP conversion were measured and plotted with an apparent Km of 0.5 μM for Rv3383c and 1.0 μM for Rv0562, with Vmax of 3.5 and 2.7 μM/min, respectively. The Michaelis-Menten curves were fit to the observed data (GraphPad 4.0, Synergy Software, Reading, PA, USA), resulting in R² values ≥ 0.95.

**RESULTS**

**POTENTIAL ISOZYMES FOR BOTH Rv3383c AND Rv3382c**

To begin investigating the hypothesized biochemical redundancy, the uncharacterized Rv3382c and Rv3383c were analyzed and compared to the rest of the Mtb genome. Rv3382c is 990 bp long and encodes a protein of 329 amino acids (AA) that has been annotated as lytB1, while Rv3383c is 1053 bp long and encodes a 350 AA protein annotated as lytB2 (Camus et al., 2002). While expression of Rv3382c did not exert any toxicity relative to expression of Rv1110 instead), culture growth was monitored every 20 min by measuring OD600.

**FUNCTIONAL ANALYSIS OF Rv3382c AND Rv1110**

LytB is a [4Fe–4S] protein that has been shown to be difficult to work with in vitro, due to complications from both oxygen corruption and substrate availability (Adami et al., 2002; Wolff et al., 2003). Accordingly, such enzymatic activity is most often demonstrated by a complementation approach (McAteer et al., 2001; Rohlich et al., 2002; Hursh and Goodman, 2003). Briefly, this utilizes a previously developed strain of E. coli in which the endogenous LytB gene (EGI1081) has been put under control of an arabinose promoter, such that bacterial growth requires either supplementation with arabinose or complementation with a functional LytB (McAteer et al., 2001). Both Rv3382c and Rv1110 were able to complement the growth of this strain of E. coli in the absence of arabinose, demonstrating functional LytB activity. Relative growth rate analysis indicates that complementation with Rv3382c results in somewhat less vigorous growth than does complementation with Rv1110, which does not appear to be due to a toxicity effect (Figure 2). This result then suggests that Rv3382c exhibits somewhat less robust LytB activity than Rv1110, which might be due to Rv3382c being 8 AA shorter at the N-terminus. However, this region does not contain any residues known to be involved in enzymatic activity, and the exact mechanism underlying reduced function of the LytB encoded by Rv3382c is unclear.

**ANALYSIS OF PUTATIVE GGPP SYNTHASES**

The production of GGPP is catalyzed by the condensation of IPP with allylic isoprenyl diphosphate acceptors, such as DMAPP (which then requires the addition of three molecules of IPP), GPP.
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FIGURE 2 Complementation assay for LytB activity. Growth curve of lytB− E. coli complemented with Rv1110 (circles) or Rv3382c (squares). These bacteria were grown to saturation, then diluted 1/100 into fresh media containing 0.5 mM IPTG. After 60 min, cultures were diluted 1/50 into media containing selective antibiotic and 0.02% glucose (to suppress endogenous LytB expression). After 220 min, cultures were diluted 1/100 in media containing selective antibiotic, but 0.2% arabinose instead of glucose, which activated expression of the endogenous LytB, to demonstrate that the Rv3382c gene itself did not have any deleterious effects (i.e., as both recombinant strains then exhibited equivalent growth).

FIGURE 3 Substrate plasticity of GGPPS. GGPPS enzymes are sometimes able to utilize a variety of substrates to synthesize the 20-carbon product, GGPP. Outlined are the various biosynthetic pathways for GGPP production in Mtb.

Table 1 Kinetic analysis of confirmed GGPP synthases.

| Enzyme   | IPP | DMAPP | GPP | FPP |
|----------|-----|-------|-----|-----|
|          | KM  | kcat  | KM  | kcat | KM  | kcat |
| Rv0562   | 1.7 ± 0.7 | 0.17 ± 0.02 | 5 ± 2 | 0.3 ± 0.2 | 2.3 ± 0.8 | 0.7 ± 0.1 | 11 ± 3 |
| Rv3383c  | 11 ± 2 | NR    | NR  | NR   | 10 ± 0.5 | 19 ± 5 |

NR, no reaction.

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other mycobacteria (Reddy et al., 2009), these were analyzed for sequences could be found not only in the MTC and but also the much more distantly related M. marinum, although it is not present in the even more distantly related M. leprae, suggesting that the isoTb operon was acquired prior to separation of the lineages leading to M. marinum and the MTC/M. canetti. We further characterized the existing polymorphisms of not only the diterpene synthases [i.e., Rv3377c and Rv3378c, as previously reported (Mann and Peters, 2012)], but also the entire isoTb operon in these mycobacteria. Notably, very few mutations are found within the unique diterpene synthase encoding genes (i.e., Rv3377c and/or Rv3378c) in the sequenced strains of Mtb (Comas et al., 2010), with none in the highly infectious Beijing subgroup. By contrast, the other species within the MTC, as well as M. canetti, all have accrued frameshift and/or non-synonymous mutations in Rv3377c or Rv3378c. The range of observed mutations in these diterpene synthases is consistent with the current view of mycobacteria evolution, with only a single non-synonymous mutation found in the more recently derived M. africanum. By contrast, the more distant relatives M. canetti and M. marinum have acquired multiple other IDH homologs. In any case, this rationale does not hold true for other mycobacteria, leaving unclear what selective pressure led to the wider conservation of Rv3383c/idsB, even upon loss of the other genes in the isoTb operon (e.g., in M. ulcerans). In the case of M. marinum, this might be due to its production of GGPP-derived carotenoids (Ichiyama et al., 1988), although M. marinum also has acquired a GGPP synthase isozyme, which is consistent with our previous speculation positoning a role for isoTb in the highly infectious nature of this human-specific pathogen (Mann and Peters, 2012).

**CONSERVATION OF GGPP SYNTHASES WITHIN THE MYCOBACTERIA**

Although the ability to produce isoTb has not been found and seems unlikely to occur outside of Mtb, it is notable that the GGPP producing enzyme found in Mtb is subject to stronger conservation ([Figure 6](#)). Strikingly, the GGPP synthase encoding Rv0562/idsB from the isoTb operon was retained even through the reductive evolution process leading to M. ulcerans where the remainder of the operon is lost ([Figure 5](#)). While the presence of a GGPP synthase is likely due to its role in production of carriers involved in cell wall synthesis, the retention of multiple GGPP synthases in these mycobacteria is striking, albeit somewhat puzzling. Rv0562/idsB is likely the ancestral GGPP synthase and is widely found in the family Mycobacteriaceae, e.g., an ortholog is present in the fast-growing, non-pathogenic saprophyte M. smegmatis. However, these mycobacteria derived from the MTC and M. marinum ancestral strain also have retained the additional GGPP synthase encoded by Rv3383c/idsB, even upon loss of the other genes in the isoTb operon (e.g., in M. ulcerans). In the case of M. marinum, this might be due to its production of GGPP-derived carotenoids (Ichiyama et al., 1988), although M. marinum also has acquired a GGPP synthase isozyme, which is consistent with our previous speculation positoning a role for isoTb in the highly infectious nature of this human-specific pathogen (Mann and Peters, 2012).

**DISCUSSION**

*Mycobacterium tuberculosis* is a persistent and deadly human pathogen whose ability to infiltrate and spread within the human immune system has led to intense interest in all aspects of its life cycle (Russell et al., 2010). Isoprenoid metabolites play a variety of roles in Mtb and other bacteria, including electron transfer components and cell wall biosynthesis (Daum et al., 2009). At least in the case of Mtb, this appears to extend to its pathogenic lifestyle (Dhiman et al., 2004; Kaur et al., 2004; Holsclaw et al., 2008; Mann et al., 2009b). In particular, the bioactive diterpenoid...
isoTb seems to contribute to early processes in Mtb infections, based on both its biological activity (Mann et al., 2009b), and the reduced proliferation of Mtb unable to produce isoTb (Pethe et al., 2004). Moreover, specific conservation of the requisite diterpene synthases Rv3377c and Rv3378c in Mtb relative to the rest of the species in the MTC (Figure 5), has led to the suggestion that isoTb may play a role in the highly infectious nature of Mtb in humans (Mann and Peters, 2012), making it a potentially human-specific pathogenic natural product.

The two diterpene synthases required for isoTb biosynthesis were isolated in a screen for genes contributing to the early infection process. However, while the identified Rv3377c and Rv3378c are found in a larger five-gene operon, the remaining three genes were not isolated in this genetic screen, despite its saturating nature (Pethe et al., 2004). Presumably, these three genes originally functioned to increase flux into and through the MEP isoprenoid pathway (Pethe et al., 2004). Given the non-essential nature of Rv3383c indicated by previously reported genetic screens (Sassetti et al., 2001; 2003; Sassetti and Rubin, 2003; Joshi et al., 2006), our results indicate that Rv3383c is functionally redundant. By contrast, Rv0562 (idsB) has been predicted to be an antimicrobial active agent (Camus et al., 2002), and our studies confirm that both Rv1110/lytB2 and Rv3382c/lytB1 exhibit such activity (Figure 2).

Given the non-essential nature of either of these genes indicated by previously reported genetic screens (Sassetti et al., 2001; 2003; Sassetti and Rubin, 2003; Joshi et al., 2006), our results indicate that Rv1110 and Rv3382c are functionally redundant. The essential nature of isoprenoid metabolism, along with the sole use of the distinct MEP pathway for precursor supply in bacteria indicates that the enzymes mediating this process might provide good targets for development of antibacterial agents (Singh et al., 2007; Eoh et al., 2009; Obiol-Pardo et al., 2011). However, it should be noted that, despite their analogous activity, the LytB paralogs share only 50% AA identity, and such divergence suggests that it may be difficult to simultaneously inhibit both gene products, i.e., to pharmacologically block this step of the MEP pathway in Mtb.

Our results further demonstrate that Mtb contains two GGPP synthases, encoded by Rv3383c/idsB and Rv0562/grcC2 (Table 1 and Figure 3). Given the non-essential nature of Rv3383c indicated by previously reported genetic screens (Sassetti et al., 2001, 2003; Sassetti and Rubin, 2003; Joshi et al., 2006), our results suggest that Rv3383c is functionally redundant.
has been found to be essential in Mtb (Sasselet et al., 2003). Its ancestral function presumably was to initiate the production of long chain isoprenylated carriers required for cell wall assembly, as this is derived from GGPP in mycobacteria other than Mtb (Becerra et al., 1994), and Rv0562 is extremely well conserved in the mycobacterial family (Figure 6). However, Mtb uses (Z/E)-FPP instead of GGPP as the precursor for cell wall isoprenylated carrier biosynthesis (Schulbach et al., 2000). Thus, particularly since Rv0562 is located just downstream of genes encoding other menaquinone biosynthetic machinery in the Mtb genome (Camus et al., 2002), we hypothesize that it may instead be required for generation of GGPP for production of this essential electron carrier, presumably as precursor to the known longer chain isoprenyl sidechain (Holcica et al., 2008). As an alternative and/or supplemental rationale for the essential nature of Rv0562, our results indicate that this gene encodes a GGPP synthase with more robust and enzymatic activity than that encoded by Rv3383c (i.e., that encoded by Rv0562 can produce GGPP from any length allylic precursor, rather than being limited to FPP as is the ID's encoded by Rv3383c).

In conclusion, our results clarify not only the role of all the genes in the operon associated with isoTb biosynthesis, but also previous metabolic, biochemical, and genetic observations regarding isoprenoid metabolism in Mtb, as described above. Nevertheless, there are questions that arise from the results reported here, such as the role of the remaining uncharacterized IDs in the MTC, and the puzzling observation of widespread conservation of Rv3383c/isdTb, presumably encoding a second GGPP synthase, in mycobacteria, which we hope to address in future studies.

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REFERENCES

Adam, P., Hadi, S., Eisenreich, W., Kaiser, J., Graevert, T., Argenti, D., et al. (2002). Biochemical aspects of isoprenoid syntheses in Mycobacterium, as evidenced by the total synthesis of 1-deoxy-9′-idothrombomycin. J. Am. Chem. Soc. 124, 12130–12133.

Baleen, A. M., Mahapatra, S., Brennan, F. J., and Crick, D. C. (2002). Identification, cloning, purification, and enzymatic characterization of Mycobacterium tuberculosis 1-deoxy-D-xylulose 5-phosphate synthase. Glycobiology 12, 811–820.

Becerra, G. S., Besra, G. S., Sievert, T., Lee, R. E., Dhiman, R. K., Born, P., and Crick, D. C. (2000). Polyprenyl diphosphate synthase from Mycobacterium tuberculosis: structural characterization of a novel prenyl lipid biosynthetic system. J. Biol. Chem. 275, 1341–1349.

Becerra, G. S., Sasselet, T., Lee, R. E., Dhiman, R. K., Born, P., and Crick, D. C. (1994). Identification of the apparent carrier in mycolic acid synthesis. Proc. Natl. Acad. Sci. U.S.A. 91, 12775–12779.

Buch, J., Gutierrez, M. C., Rosas Magallanes, V., Bazos, J., Gospod, B., Nerymilis, O., et al. (2007). Contribution of horizontally acquired genomic islands to the evolution of the tubercle bacilli. Mol. Biol. Evol. 24, 1863–1872.

Burk, C., and Croteau, R. (2002). Interaction with the small subunit of geranylgeranyl diphosphate synthase modifies the chain length specificity of geranylgeranyl diphosphate synthase to produce geranyl diphosphate. J. Biol. Chem. 277, 5414–5419.

Camus, I., Chakravorti, I., Small, P. M., Galagan, J., Neumann, F., Kremer, K., et al. (2010). Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily conserved. Nat. Genet. 42, 499–503.

Crick, D. C., Schulbach, M. C., Zink, E. E., Macchia, M., Barontini, S., Becerra, G. S., et al. (2003). Polyprenyl phosphate biosynthesis in Mycobacterium tuberculosis and Mycobacterium avium. J. Bacteriol. 185, 5771–5778.

Duan, M., Herrmann, S., Wilkinson, B., and Bachhold, A. (2009). Genes and enzymes involved in bacterial isoprenoid biosynthesis. Curr. Opin. Chem. Biol. 13, 180–188.

Dhiman, R. K., Schulbach, M. C., Mahapatra, S., Raulard, A. K., Vosa, V., Bornan, P. J., et al. (2004). Identification of a novel class of omega,E,Z-unsaturated diphosphate synthase from Mycobacterium tuberculosis. J. Biol. Chem. 279, 1140–1147.

Eiglmeier, K., et al. (2002). A complete genome sequence of Mycobacterium avium. Proc. Natl. Acad. Sci. U.S.A. 99, 182, 10676–10681.

Eiglmeier, K., Frigui, W., Valenti, P., Murray, J., Hirschberg, R., Lipatov, M., Small, P. M., Sheffer, H., Niemann, S., Homolka, S., et al. (2008). High functional diversity in Mycobacterium tuberculosis, so driven by genetic drift and human demographics. Proc. Biol. Sci. 275, 101371. doi:10.1098/rspb.2008.1051

Holcica, C. M., Sogi, K. M., Gilmour, S. A., Scholl, M. W., LeDell, M. D., Bertoni, C. R., et al. (2008). Structural characterization of a novel sulfated menaquinone produced by Mycobacterium tuberculosis MEF (2C-methyl-d-isoserine-4-phosphate) pathway as a new drug target. Tuberculosis (Edinb.) 88, 1–11.

Hernandez, B., Lipani, M., Small, P. M., Sheffer, H., Niemann, S., Homolka, S., et al. (2008). High functional diversity in Mycobacterium tuberculosis, so driven by genetic drift and human demographics. Proc. Biol. Sci. 275, 101371. doi:10.1098/rspb.2008.1051

Ishikawa, T., Itoman, T., Oda, T., and Sakata, Y. (2002). Biosynthesis of 2-(Z)-farnesyl diphosphate synthase from Mycobacterium tuberculosis. J. Biol. Chem. 277, 1140–1147.

Kaiser, J., Grawert, T., Arigoni, M., et al. (2007). Conservation of GGPP synthases in the MTC and closely related mycobacteria. Mol. Biol. Evol. 24, 1863–1872.
Mann, F. M., Xu, M., Chen, X., Fulton, M. L., Peters, R. J., and Sutter, R. B. (2010). Synthesis of (+/-)-monothiolated (methoxybenzylidene, reduced structure of edaxadiene) and (+/-)-tuberculosinol. Org. Lett. 12, 2620–2623.

Mckee, S. A., Donahue, M. C., Mckee, N., and Masters, M. (2001). The lytB gene of Escherichia coli is essential and specifies a product needed for intracellular biosynthesis. J. Bacteriol. 183, 7403–7407.

Nakano, C., Okima, T., Sato, T., Dairi, T., and Hoshino, T. (2011). Characterization of the Rv0989c gene product, a new deacetylase synthase for producing tuberculosinol and (+/-)-tuberculosinol (novel-borrel, from the Mycobacterium tuberculosis Rv0989c genome. Biochem. Biophys. Res. Commun. 396, 75–81.

Nakano, C., Sato, T., and Hoshino, T. (2010b). Structural analysis of the product produced by Rv0989c gene product from Mycobacterium tuberculosis. Kyo Bun Gakkaishi 89, 247–254.

Oishi-Parada, C., Rubio-Martinez, J., and Imperial, S. (2011). The methylenedithiol phosphate (MEP) pathway for intracellular biosynthesis is a target for the development of new drugs against tuberculosis. Curr. Med. Chem. 18, 1322–1336.

Peth, K., Wieson, D. L., Akutsu, S., Anderson, J., Wang, C., and Russell, D. G. (2006). Localization of Mycobacterium tuberculosis mutase-defective in the arrest of phagosome maturation. Proc. Natl. Acad. Sci. U.S.A. 103, 13642–13647.

Pugs, L., Kirby, J., Kremling, D. J., and Albert, T. (2010). Diterpenoid production in Mycobacterium marinum. FEBS Lett. 577, 558–565.

Rudd, T. R., Biley, R., Wyman, F., Montgomery, P., Decaprio, D., Engels, R., et al. (2009). TB-database an integrated platform for tuberculosis research. Nucleic Acids Res. 37, D495–D500.

Rohde, K., Yate, R. M., Purdy, G. E., Reddy, T. B., Riley, R., Wymore, R., et al. (2009). TB database: implications for tuberculosis. FEBS Lett. 585, 549–554.

Rohde, K., Yate, R. M., Purdy, G. E., Reddy, T. B., Riley, R., Wymore, R., et al. (2009). TB database: implications for tuberculosis. FEBS Lett. 585, 549–554.

Russell, D. G. (2007). Who puts the tubercle in tuberculosis? Nat. Rev. Microbiol. 5, 39–47.

Russell, D. G., Barry, C. E. III, and Hulten, E. J. (2001). Comprehensive identification of conditionally essential genes in Mycobacteria. Proc. Natl. Acad. Sci. U.S.A. 98, 12712–12717.

Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003). Identification of a novel pathway for the early steps in mycobacterial intracellular survival during infection. Proc. Natl. Acad. Sci. U.S.A. 100, 12899–12994.

Schmidt, A., and Gardeshnak, J. (2011). Cloning and characterization of two different types of genotypic diphosphate synthase from Nocardia sp. (Pura abces). Phytochemistry 72, 49–57.

Shibabach, M. C., Brennan, P. J., and Crick, D. C. (2000). Identification of a short (C15) chain Z-isoprenyl diphosphate synthase and a homologue long (C30) chain Z-isoprenyl diphosphate synthase in Mycobacterium tuberculosis. J. Biol. Chem. 275, 22876–22881.

Singh, N., Chavez, G., Avery, M. A., and MacIntyre, C. B. (2007). Targeting the mevalonate pathway (MEP) pathway for novel antimalarials, antibacterial and herbicidal drug discovery: inhibition of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) enzyme. Curr. Pharm. Des. 13, 1161–1175.

Smith, P. H. A., and Solak, R. B. (1973). Numerical Taxonomy: The Principles and Practice of Numerical Classification. San Francisco W. H. Freeman.

Stinear, T. P., Caron, C. A., and Skennern, E. J. (2010). Synthase enables a structural transition of the Mycobacterium tuberculosis-iso-tuberculosinol operon.