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Magnesium depletion triggers production of an immune modulating diterpenoid in *Mycobacterium tuberculosis*

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Summary

Mycobacterium tuberculosis (*Mtb*) is the causative agent of the human disease Tuberculosis, and remains a worldwide health threat responsible for ~1.7 million deaths annually. During infection, *Mtb* prevents acidification of the engulfing phagosome, thus blocking endocytic progression and eventually leading to stable residence. The diterpenoid metabolite isotuberculosinol (isoTb) exhibits biological activity indicative of a role in this early arrest of phagosome maturation. Presumably, isoTb production should be induced by phagosomal entry. However, the relevant enzymatic genes are not transcriptionally upregulated during engulfment. Previous examination of the initial biosynthetic enzyme (Rv3377c/MtHPS) involved in isoTb biosynthesis revealed striking inhibition by its Mg²⁺ co-factor, leading to the hypothesis that the depletion of Mg²⁺ observed upon phagosomal engulfment may act to trigger isoTb biosynthesis. While *Mtb* is typically grown in relatively high levels of Mg²⁺ (0.43 mM), shifting *Mtb* to media with phagosomal levels (0.1 mM) led to a significant (~10-fold) increase in accumulation of the MtHPS product, halimadienyl diphosphate, as well as easily detectable amounts of the derived bioactive isoTb. These results demonstrate isoTb production by *Mtb* specifically under conditions that mimic phagosomal cation concentrations, and further support a role for isoTb in the *Mtb* infection process.

Introduction

First described in 1882, *Mycobacterium tuberculosis* (*Mtb*), the main pathogen responsible for the human disease Tuberculosis, remains a major health concern into the 21st century. It has been estimated that up to one-third of the global population harbors the bacteria, with approximately 1.7 million deaths due to Tuberculosis yearly (Russell *et al.*, 2010). Further, the recent emergence of multi- and extremely drug resistance strains highlights the continued relevance of this pernicious human pathogen (Chiang *et al.*, 2010). The complete mechanism of pathogenesis is unknown, but likely involves a multi-factorial attack of the immune system (Deretic *et al.*, 2006).

Mtb resides within macrophage phagosome compartments that are arrested at an early stage of maturation that prevents delivery of *Mtb* to bactericidal phagolysosomes (Vergne *et al.*, 2004). Therefore, a key step in the *Mtb* infection process is blocking endocytic progression of the bacteria containing phagosome. Although there is clear evidence that mycobacterial specific cell surface lipids play a role in halting phagosome maturation (Guenin-Mace *et al.*, 2009, Chua *et al.*, 2004), it seems likely that there are multiple factors affecting this critical aspect of the *Mtb* life-cycle (Russell, 2007), as independent genetic screens have uncovered

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non-overlapping sets of genes required for pathogenesis (Sasseti & Rubin, 2003, Joshi *et al.*, 2006, Rengarajan *et al.*, 2005).

The only genetic screen focused on *Mtb* genes involved in early infection highlighted a role for two previously unstudied genes, Rv3377c and Rv3378c, found in an operon that seemed to be involved in isoprenoid/terpenoid biosynthesis (Petthe *et al.*, 2004). This was verified by subsequent biochemical analysis, as Rv3377c was demonstrated to encode a class II diterpene cyclase that carries out bicyclization and rearrangement of the universal diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP) to produce halimadienyl diphosphate (HPP) (Nakano *et al.*, 2005). Subsequently, it has been reported that the enzyme encoded by Rv3378c acts upon HPP to produce either a mixture of the corresponding primary and tertiary alcohols [termed tuberculosinol and isotuberculosinol, respectively (Nakano, 2005)], or a novel tricyclic diterpene (Mann *et al.*, 2009b). Recent results demonstrate that the true enzymatic product is isotuberculosinol (isoTb) (Spangler *et al.*, 2010, Mangel *et al.*, 2010). Notably, in bead-based phagocytosis assays pure isoTb halted acidification at a pH 0.5 units above that of the control (Mann *et al.*, 2009b), matching the defect observed with the corresponding *Mtb* Rv3377c and Rv3378c mutants, which also were impaired in their ability to replicate in macrophage cell culture (Petthe *et al.*, 2004).

The biological activity of isoTb strongly suggests a role in inhibiting the phagosome maturation process and, while a recent report demonstrates that it is possible to detect isoTb from *Mtb* (Prach *et al.*, 2010), the levels of isoTb found in *Mtb* grown in the standard 7H9 media are quite low, suggesting the possibility that isoTb biosynthesis might need to be increased upon phagosomal engulfment. However, genome-wide pre- and post-infection microarrays have failed to find changes in the transcription of Rv3377c and Rv3378c upon macrophage infection (Rohde *et al.*, 2007, Waddell & Butcher, 2007, Stewart *et al.*, 2005).

Our recent kinetic analysis of the HPP synthase encoded by Rv3377c (MtHPS) uncovered an intriguing biphasic response of enzymatic activity to its divalent magnesium ion (Mg^{2+}) co-factor, which led us to hypothesize that this might act as a physiologically relevant regulatory mechanism (Mann *et al.*, 2009a). Specifically, MtHPS exhibits optimal activity in the presence of 0.1 mM Mg^{2+} , and even slightly higher levels dramatically reduce its activity (i.e. 60% reduction at 0.5 mM and above). Intriguingly, during *Mtb* entry the bacteria transition from the high- μ M levels of Mg^{2+} observed in oral and lung mucus (Effros *et al.*, 2005, Shpitzer *et al.*, 2007) to the 10–50 μ M levels found in macrophage phagosomes, at least those containing *Salmonella* (Garcia-del Portillo *et al.*, 1992), which is close to the optimal level for MtHPS activity *in vitro*. In addition, studies of other intracellular pathogens, such as *Brucella suis*, further support the low concentration of magnesium in the phagosome (Lavigne *et al.*, 2005). Indeed, it is hypothesized that *Mtb* shares a parallel strategy for surviving the initial shock of uptake into this depleted environment with *Salmonella* and *Brucella* (Lavigne *et al.*, 2005, Buchmeier *et al.*, 2000), although such depletion of Mg^{2+} has not been directly measured with *Mtb* containing phagosomes. Nevertheless, we hypothesized that MtHPS activity, representing the committed step in isoTb biosynthesis (Figure 1), is induced by this depletion of Mg^{2+} (Mann *et al.*, 2009a). This also was based in part on the observation of such Mg^{2+} -dependent regulation of plant class II diterpene cyclases (Prisic & Peters, 2007, Mann *et al.*, 2010), which are distant homologs of MtHPS (Cao, 2010). Indeed, active site mutant analysis (Nakano & Hoshino, 2009) and shared susceptibility to mechanism based inhibitors indicate that MtHPS catalysis is analogous to that mediated by these plant enzymes (Mann *et al.*, 2009a). This hypothesis also might then explain previous difficulties in detecting isoTb and halimadienol from *Mtb*, which is typically grown rich media containing higher amounts (~0.43 mM) of Mg^{2+} (Wards, 1996).

Here we have tested and verified this hypothesis in *Mtb*. By understanding the metabolism and regulation of this pathway, we hope to clarify the role of isoTb in the *Mtb* infection process, and shed light on this factor assisting *Mtb* attack on the human immune system.

Experimental procedures

General

Geranylgeranyl diphosphate was purchased from Isoprenoids, LC (Tampa, FL). All other chemicals were purchased from Fisher Scientific (St. Louis, MO) unless otherwise noted. Molecular biology reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. High Performance Liquid Chromatography (HPLC) separations were performed on an Agilent (Santa Clara, CA) 1100 instrument equipped with automated sample injector and fraction collector, as well as diode array detector and heated column compartment. GC-MS analysis was performed on a Varian 3900 GC with Saturn 2100T ion-trap detector (Palo Alto, CA) using an Agilent HP-1MS column (Santa Clara, CA).

Measurement of halimadienyl diphosphate in recombinant *E. coli*

To endow *E. coli* with the ability to produce HPP, the previously described MBP-Rv3377c fusion from a pTH1/DEST:Rv3377c expression vector (Mann et al., 2009b) was cloned into a previously described pACYC-Duet vector (Novagen, Merck Corp.), that already contained a GGPP synthase from *Abies grandis* (Burke & Croteau, 2002) in the second multiple cloning site (Cyr *et al.*, 2007). Specifically, MBP-Rv3377c was cloned into the first multiple cloning site of this pGG vector using 5' NcoI and 3' HindIII restriction sites, which were introduced into MBP-Rv3377c by PCR, creating a pGG/MBP-Rv3377c co-expression vector. *E. coli* C41 (Lucigen, Middleton, WI) was transformed with this vector and selectively grown on NZY agar plates or in TB liquid media (12 g/L casein, 24 g/L yeast extract, 8 mL/L glycerol, 100 mM phosphate buffer, pH 7.0). These recombinant cells were grown in modified liquid TB media (containing 0.43 mM MgCl₂) to OD₆₀₀ ~ 0.8, with cultures then moved to 16 °C for 1 hr, followed by induction with 0.5 mM IPTG, with an additional 14–16 hrs of fermentation. Control cultures were allowed to grow for the entire experimental period. The effect of Mg²⁺ depletion was assessed by pelleting 50-mL cultures at 4 °C. These cells were washed with 0.1 M phosphate buffer, pH 7, and then resuspended in 50-mL fresh TB culture media supplemented either 0.1 or 0.4 mM MgCl₂, and fermentation continued at 16 °C. Triplicate time points were taken prior to this media shift, and then 30 min., 1 hr, 6 hrs, and 24 hrs following the shift in media (i.e., transfer), by pelleting cells from a 50-mL aliquot and extracting the resulting clarified media with an equal volume of hexane, which was then dried to completion and resuspended in 100 µL fresh hexane for analysis. Pellets from each time point were weighed and analyzed for overexpression of MthPS via denaturing polyacrylamide gel electrophoresis and subsequent Coomassie blue staining. Bands were quantified and subject to statistical analysis.

Construction of a ΔRv3377-78c strain and Rv3377-78c complementing plasmid

A ΔRv3377-78c strain was constructed by in *Mtb* CDC1551 by an allelic exchange method using the pYUB854 plasmid (Bardarov *et al.*, 2002). The *Mtb rpsL* (Rv0682) gene was PCR amplified and ligated into the PacI site of pYUB854 for counter selecting purposes (Sander *et al.*, 1995). For allelic exchange, ~600-bp DNA fragments corresponding to the upstream and downstream region of the Rv3377–Rv3378 locus were cloned into pYUB854 to flank the hygromycin resistance gene. The final plasmid was electroporated into streptomycin-resistant *Mtb* CDC1551. Hygromycin-resistant and streptomycin-resistant clones were screened by PCR and Southern blot to confirm loss of Rv3377c and Rv3378c. Rv3377c and Rv3378c were cloned from *Mtb* CDC1551, recombined as a single transcriptional unit into pMV361 (Stover *et al.*, 1992) under control of a blaF promoter (Lim *et al.*, 1995), and

verified by complete gene sequencing. The resulting plasmid was used to complement the $\Delta Rv3377-78c$ *Mtb* CDC1551 by transformation following published protocols (Snapper *et al.*, 1988).

Measurement of isotuberculosinol in *Mtb*

Mtb was grown using 7H9 media (0.5 g/L ammonium sulfate, 0.5 g/L L-glutamic acid, 0.1 g/L sodium citrate, 1.0 mg/L pyridoxine, 0.5 mg/L biotin, 2.5 g/L dibasic sodium phosphate, 1.0 g/L monobasic potassium phosphate, 0.04 g/L ferric ammonium citrate, 0.5 mg/L calcium chloride, 1.0 mg/L zinc sulfate, 1.0 copper sulfate, 8.5 g/L sodium chloride, 50 g/L bovine albumin, 20 g/L dextrose, 0.03 g/L catalase, pH 6.6, and typically 0.05 g/L magnesium sulfate). Modified liquid media was made up by altering the amount of $MgSO_4$ to achieve concentrations of 0.1 mM Mg^{2+} and/or by the addition of Mn^{2+} 2.5 mM ($MnCl_2 \cdot 4H_2O$). Specifically, to make up '7H9-low' media containing only 0.1 mM Mg^{2+} with the addition of 2.5 mM Mn^{2+} , and '7H9- Mn^{2+} ' media wherein the only change is the addition of 2.5 mM Mn^{2+} (as the counter-anion does not affect MtHPS activity; Table S1). Bacteria ($\sim 1 \times 10^{11}$ cfu) were inoculated into 200 mL of media and grown for 2 weeks. Cells were then pelleted and resuspended in 7H9 or experimental media conditions and allowed to grow for 2 more weeks. Media was then extracted with equivalent volumes of hexane and dried. Extracts were resuspended in 1 mL fresh hexanes for initial analysis via gas chromatography-mass spectroscopy (GC-MS) against previously prepared authentic standards (Mann et al., 2009a, Mann et al., 2009b). Crude extracts proved too complex for quantification, and samples were dried and resuspended in 1:1 (v/v) mixture of acetonitrile:water for fractionation by HPLC utilizing C-8 reverse-phase analytical column (Kromasil, Brewster, NY) with a flow rate of 0.2 mL/min at 40 °C against a 50–100% acetonitrile (ACN) gradient (0–15 min, 50% ACN 0.5 mL/min; 15–30 min 50–100% ACN 0.1 mL/min; 30–39.9 min 100% ACN 0.5 mL/min; 39.9–40.0 50% ACN). The fractionated extracts were dried and resuspended in hexane for GC-MS analysis. Extracts were normalized against endogenous menaquinone concentrations, which were quantified as varying less than 10% by comparison to known concentrations of an authentic standard (Table S2) (Sigma-Aldrich, St. Louis, MO), and the concentrations of isoTb, halimadienol, and geranylgeraniol diterpenoids were quantified via total ion chromatograph against a standard curve constructed with the diterpene cembrene (Sigma-Aldrich) using amounts ranging from 10 – 500 ng/ μ L. Trace metabolites were quantified via extracted ion chromatographs of ions 119, 191, 290.

Transcriptional analysis of *Rv3377c* and *Rv3378c* in *Mtb*

Mtb CDC1551, *Mtb* CDC1551 $\Delta 3377-78c$, and *Mtb* CDC1551 $\Delta 3377-78c$ complemented strains were grown in the previously described experimental conditions, and 4 mL of bacterial culture were pelleted via centrifugation for RNA extraction. Bacterial pellets were resuspended in 1 mL GTC buffer (4M guanidine thiocyanate, 0.5% sodium-lauryl sarcosine, 25 mM sodium citrate, 0.1 M β -mercaptoethanol) and frozen at -80 °C for 16 hours. Cultures were thawed and pelleted before washing in PBS buffer (0.15 M sodium chloride, 10 mM dibasic sodium phosphate, 1.75 mM monobasic sodium phosphate, and 0.1% tween-20, pH to 7.4). Washed pellets were resuspended in 200 μ L lysozyme (10 mg/mL) and incubated at 25 °C for 15 minutes. Hot (65 °C) Trizol (750 μ L) was added before cellular disruption with ~ 0.5 mL glass beads (0.1 mm) for 2 minutes by beadbeating. Lysates were phase separated by adding 200 μ L chloroform and centrifuged for 15 minutes. 500 μ L ethyl alcohol was added to the aqueous phase and RNA was isolated via the Qiagen RNeasy kit (Valencia, CA) with elution by 2×50 μ L sterile water. The resulting RNA was treated with Ambion TurboDNase Free according to manufacturer protocols (Austin, TX) before quantification. Equivalent concentrations of RNA (600 ng) were converted to cDNA via the Iscript reaction as per manufacturer protocols (BioRad, Hercules, CA), and 2 μ L of each

cDNA reaction was amplified with gene-specific primers for Rv3377c or Rv3378c in 25 μ L reactions using Invitrogen Accuprime Taq (Carlsbad, CA) and recommended polymerase chain reaction conditions (95°C 5 min, followed by 95 °C 30 sec, 60 °C 2 min, 72 °C 3 min for 45 cycles, 72 °C 10 minutes). Transcription of endogenous sigma factor A (SigA, Rv2703) was measured for normalization of gene transcription. PCR products were separated on a 1.5% agarose gel containing ethidium bromide (Figure S1) and visualized with UV irradiation for quantification and statistical analysis (Table S3).

Results

As an initial test of our hypothesis that external Mg^{2+} levels can regulate MthPS activity inside bacterial cells, we engineered *E. coli* for the production of HPP. Specifically, *E. coli* transformed with a single plasmid enabling inducible co-expression of a GGPP synthase and MthPS expressed as fusion protein with MBP. Following induction, this recombinant *E. coli* was subjected to changes in environmental Mg^{2+} levels designed to mimic the conditions that *Mtb* is believed to experience when it is engulfed by the phagosome. In particular, the cells were grown in media containing 0.43 mM Mg^{2+} , induced, and then pelleted ~16 hrs after induction. These cell pellets were washed, and then transferred to media with either at a high (0.4 mM) or low (0.1 mM) level of Mg^{2+} , with the latter approximating that found in phagosomes (Garcia-del Portillo et al., 1992), and equivalent to the optimal concentration for MthPS activity *in vitro* (Mann et al., 2009a). Cultures transferred to high (0.43 mM) Mg^{2+} media exhibited reduced accumulation of HPP-derived HaOH compared to cultures transferred to low (0.1 mM) Mg^{2+} (Figure 2A). This appears to occur due to increased flux of GGPP through MBP-MthPS, as concentrations of GGPP-derived GGOH are low compared to concentrations in cultures transferred to high Mg^{2+} media (Figure 2B), yet the relative overall concentration of diterpenoid metabolites is comparable between populations. During this time, no changes in MBP-MthPS protein levels were noted. This confirms that, at least in *E. coli*, changes in environmental Mg^{2+} levels do influence HPP production, presumably via a direct effect on intrinsic MthPS activity within the bacteria.

It is well understood that *Mtb* and *E. coli* differ drastically, especially in metabolism, growth, and cell wall constituents (Kumar & Robbins, 2007). Because of these differences, it was imperative to determine if similar Mg^{2+} depletion could also directly influence internal enzymatic activity and metabolic flux in *Mtb*. Furthermore, at the time this study was initiated, the expected HPP intermediate and bioactive isoTb end product had not yet been detected in *Mtb* itself. Notably, the preferred divalent cation for the enzyme encoded by Rv3378c that converts HPP to isoTb is Mn^{2+} (Mann et al., 2009b), which might also limit isoTb production. To address these latter two issues, *Mtb* was grown in 7H9 media containing either the standard Mg^{2+} concentration of 0.43 mM, in the absence or presence of 2.5 mM Mn^{2+} (7H9 and 7H9- Mn^{2+} , respectively), or 7H9 media containing only 0.1 mM Mg^{2+} as well as 2.5 mM Mn^{2+} (7H9-low). After growth, the bacteria and media were extracted with organic solvent, which was then analyzed for the presence of the precursor GGPP (detected as geranylgeraniol, GGOH), the MthPS derived intermediate HPP (detected as halimadienol, HaOH), and the bioactive end product isoTb (Table 1). In these extracts, isoTb was not found in *Mtb* grown in unmodified 7H9 media, although small amounts of HPP derived HaOH were detected, indicating that isoTb levels may be lower than our detection limit (i.e. less than 20 ng/L bacterial culture). However, isoTb was easily detected in *Mtb* grown in 0.1 mM Mg^{2+} containing 7H9 media supplemented with 2.5 mM Mn^{2+} , although these cells also were apparently depleted of GGPP, suggesting limitations exerted by exhaustion of this precursor. Conversely, addition of Mn^{2+} to 7H9 media led to the loss of HPP production, with isoTb also not detected, although considerably larger amounts of GGPP derived GGOH were observed. This preliminary data suggested environmental control of isoTb production occurs via regulation of MthPS activity, which is

easily inhibited by Mg^{2+} concentrations over 0.1 mM and cannot utilize Mn^{2+} (Nakano & Hoshino, 2009)(Table S1), while the GGPP and isoTb synthases are able to utilize Mn^{2+} in place of Mg^{2+} .

To directly test our hypothesis that external Mg^{2+} levels can regulate MtHPS activity and, hence, isoTb production in *Mtb* itself, the mycobacteria was grown in unmodified 7H9 media and transferred/shifted by pelleting and resuspending either back into unmodified 7H9 media or into low Mg^{2+} 7H9 media supplemented with Mn^{2+} (7H9-low). Strikingly, isoTb was easily detected when *Mtb* was grown in unmodified 7H9 media and then transferred to 7H9-low media, although only trace amounts were detected when the bacteria was shifted back to 7H9 media, indicating ≥ 100 -fold increase in this metabolite, to an easily detectable 3–5 $\mu\text{g/L}$ (Figure 3). This drastic increase in isoTb production presumably occurs due to the observed ~ 10 -fold increase in intermediate HPP production, and maintenance of GGPP precursor levels (Figure 4), as Rv3377c and Rv3378c transcript levels do not change (Table S3).

To further verify the role of Rv3377c and Rv3378c in isoTb production, as well as the observed biochemical regulation, the key Mg^{2+} depletion growth experiments were repeated with both wild-type *Mtb* CDC1551 and a derived $\Delta\text{Rv3377-78c}$ strain, which was also further complemented with a plasmid expressing Rv3377c and Rv3378c under control of a constitutive promoter. Specifically, these three *Mtb* variants were grown in unmodified 7H9 media, then pelleted and resuspended either back into unmodified 7H9 media or into 7H9-low media. As expected, the *Mtb* $\Delta\text{Rv3377-78c}$ mutant did not synthesize HPP or isoTb under any conditions, demonstrating that the enzymes encoded by Rv3377c and Rv3378c are required for such biosynthesis. Conversely, the complemented strain exhibited similar induction of isoTb production by the shift to 7H9-low, but not 7H9, as wild type *Mtb*, indicating these genes are responsible for production of these diterpenoid metabolites in *Mtb*, consistent with previously reported analysis using a *Mycobacterium smegmatis* expression system (Prach et al., 2010). In addition, these results are consistent with a biochemical mechanism by which Mg^{2+} depletion triggers isoTB production.

Discussion

The biological activity exhibited by the *Mtb* diterpenoid metabolite isoTb is strongly indicative of a role early in the infection process. Specifically, it is involved in arresting initial acidification of the phagosomal compartment. Differences in pH profile are evident within 30 min. in phagosomes containing *Mtb* lacking the relevant enzymatic genes, Rv3377c and Rv3378c, relative to those containing wild-type *Mtb* (Pethe et al., 2004), and within 10 min. in phagosomes fed isoTb coated versus control beads (Mann et al., 2009b). These effects imply that isoTb should be either constitutively produced at physiological relevant levels or rapidly biosynthesized upon phagosomal uptake. However, while transcription of Rv3377c and Rv3378c is not increased upon phagosome engulfment (Rohde et al., 2007, Waddell & Butcher, 2007, Stewart et al., 2005), indicating constitutive expression of the biosynthetic machinery, and isoTb production by *Mtb* has been reported (Prach et al., 2010), as demonstrated here isoTb is only found at very low levels in *Mtb* grown in the standard 7H9 media.

The Rv3377c encoded MtHPS catalyzes the committed step in isoTb biosynthesis (Figure 1), and, hence, controls flux into isoTb production. Intriguingly, MtHPS activity is optimal at a low (0.1 mM) concentration of Mg^{2+} (Mann et al., 2009a), which is close to the levels found in the phagosome (Garcia-del Portillo et al., 1992), while MtHPS activity is significantly reduced at higher concentrations of Mg^{2+} , particularly at levels ≥ 0.5 mM, which is similar to what is found in oral and lung mucus (Effros et al., 2005, Shpitzer et al.,

2007). In addition, internal bacterial cation concentrations have been shown to change to reflect environmental levels (Hurwitz & Rosano, 1967), and in-depth studies on *Mycobacterium tuberculosis*, *avium*, and *smegmatis* indicate that the concentrations of several cations in these bacteria are reduced after phagosomal engulfment, although Mg^{2+} itself was not detectable by the methods used (Wagner *et al.*, 2005a, Wagner *et al.*, 2005b). Nevertheless, potential transporters and chelators of Mg^{2+} from *Mtb* have been characterized and implicated in bacterial survival and pathogenesis (Blanc-Potard & Lafay, 2003, Buchmeier *et al.*, 2000, Agranoff & Krishna, 2004, Wagner *et al.*, 2005b), suggesting that depletion of Mg^{2+} in the phagosome may play a role in regulating pathogenesis. Indeed, other intracellular bacterial pathogens such as *Salmonella* and *Brucella* have been shown to respond to Mg^{2+} -depletion levels similar to those utilized here (Lavigne *et al.*, 2005, Rang *et al.*, 2007) and genes believed to be involved in intraphagosomal survival and nutrient scavenging in *Salmonella* and *Brucella* are also found in *Mtb* (Buchmeier *et al.*, 2000, Lavigne *et al.*, 2005, Rang *et al.*, 2007). Thus, we hypothesized that the Mg^{2+} depletion occurring upon phagosomal uptake provides a biochemical regulatory mechanism that activates MtHPS and triggers isoTb biosynthesis (Mann *et al.*, 2009a).

The results reported here demonstrate that Mg^{2+} depletion does trigger isoTb production in *Mtb*. Preliminary studies in *E. coli* engineered to produce the HPP product of MtHPS demonstrated that changes in external/environmental Mg^{2+} levels can rapidly regulate metabolic flux within bacteria (Figure 2), despite no change in MtHPS protein levels. Studies with *Mtb* itself further supported Mg^{2+} -depletion triggered production of isoTb via a biochemical mechanism (Table 1). In particular, transferring *Mtb* from high Mg^{2+} media to media containing 'phagosome-like' low Mg^{2+} levels, triggered not only a ~10-fold increase in HPP levels, but also the production of easily measureable isoTb (Figure 3), with an overall ~20-fold increase in biosynthesized diterpenoids (Figure 4). This was observed regardless of upstream promoter (native or recombinant), consistent with the lack of effect from Mg^{2+} -depletion on Rv3377c and Rv3378c transcripts in wild type *Mtb* (Figure S1), and indicative of a biochemical regulatory mechanism. Notably, continuous growth at low concentrations of Mg^{2+} failed to result in a similar chemotype, as *Mtb* simply grown in a low Mg^{2+} environment do produce a small amount of HPP and isoTb, but deplete GGPP precursor levels in the process (Table 1). This indicates that it is not simply low Mg^{2+} levels, but the depletion of Mg^{2+} that occurs during phagocytosis which enables flux towards such bioactive diterpenoid metabolism, triggering production of the immune modulating isoTb (i.e., via activation of MtHPS activity), as well as increasing overall flux, providing a physiologically relevant regulatory mechanism, and further supporting a role for isoTb in the *Mtb* infection process (Figure 5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Isotuberculosinol biosynthesis

Synthesis of isotuberculosinol proceeds via the cyclization of geranylgeranyl diphosphate (GGPP) to halimadienyl diphosphate (HPP) via the MtHPS encoded by Rv3377c, which is further transformed to isotuberculosinol (isoTb) via the enzyme encoded by Rv3378c.

Figure 2. Magnesium depletion triggers increased HPP production by MtHPS in *E. coli*
 Production of diterpenoid metabolites in *E. coli* expressing a GGPP synthase and MtHPS.
 Legend: High Mg^{2+} (0.43 mM) (open circles), Low Mg^{2+} (filled circles). Shown is the average, with error bars indicating the standard deviation, of two independent biological samples.

- A.** Concentration of GGPP per gram of bacterial fresh weight over time.
- B.** Concentration of HPP per gram of bacterial fresh weight over time.

Figure 3. Biosynthesis of isoTb is triggered by Mg²⁺ depletion

Selected ion ($m/z = 119 + 191 + 290$) chromatogram from GC-MS analysis of extracted diterpenoid metabolites from *M. tuberculosis* H37Rv grown in 7H9 media and transferred into new 7H9 media containing either the normal high (0.43 mM) or low (0.1 mM) concentration of Mg²⁺ compared to isoTb standard reference chromatogram (as indicated). The chromatograms are vertically offset for clarity, but those of the extracts from high and low [Mg²⁺] exposed Mtb cultures are shown on the same absolute scale.

Figure 4. Magnesium depletion triggers diterpenoid metabolism in *Mtb*

Mtb CDC1551 grown in 7H9 media for 4 weeks (“7H9”) or grown in 7H9 for 2 weeks and new 7H9 media for 2 weeks (“7H9→7H9”) show distinctly different production profiles of diterpenoid metabolites HPP (detected here as the derived HaOH) and IsoTb from bacteria subject to growth in 7H9 and subsequent shift to 7H9 containing 0.1 mM Mg²⁺ and 2.5 mM Mn²⁺ (“7H9→Low”). However, the change in environmental media conditions does not appear to effect availability of the precursor molecule, GGPP (detected here as the derived GGOH). (N.D. = not detected, L.D. = concentrations at or below limit of positive identification, 20 ng/L culture). Shown is the average, with error bars indicating the standard deviation, of two independent biological samples.

Figure 5. Proposed regulation of isotuberculosinol biosynthesis during phagosomal engulfment
 During infection, *Mtb* (purple) is compartmentalized within the phagosome (light grey) of the macrophage (dark grey). IsoTb is known to interfere with early stages of phagosomal maturation, which prevents later lysosomal (greyscale) fusion and bactericidal activity. IsoTb biosynthesis is inhibited by high Mg²⁺ concentration until phagosomal engulfment, when the bacteria are subjected to a shift to a low Mg²⁺ environment, releasing inhibition of the committed step of isoTb biosynthesis.

Table 1

Effect of divalent cations on isoTb production by *Mtb*. CDC1551 was grown in the indicated media (7H9, which contains 0.43 mM Mg^{2+} ; 7H9- Mn^{2+} was supplemented with 2.5 mM Mn^{2+} ; while 7H9-low contains only 0.1 mM Mg^{2+} , although it also was supplemented with 2.5 mM Mn^{2+}). Production of the diterpenoid metabolites GGPP, detected as the derived geranylgeraniol (GGOH), and HPP, detected as the derived halimadienol (HaOH), as well as isotuberculosinol (isoTb), were analyzed via comparison to prepared standards and normalized against endogenous menaquinone concentrations.

<u>Media</u>	<u>Metabolite Concentration (μg/L)</u>		
	<u>GGOH</u>	<u>HaOH</u>	<u>IsoTb</u>
7H9	+	+	N.D.
7H9- Mn^{2+}	++	N.D.	N.D.
7H9-low	N.D.	+	+

(N.D. = not detected; + indicates levels of production over 0.25 μg/L, ++ indicates levels of production over 0.75 μg/L). The limit of detection for this experiment is 20 ng/L culture.