

Ppm1-Encoded Polyprenyl Monophosphomannose Synthase Activity Is Essential for Lipoglycan Synthesis and Survival in Mycobacteria

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Abstract

The biosynthesis of mycobacterial mannose-containing lipoglycans, such as lipomannan (LM) and the immunomodulator lipoarabinomannan (LAM), is carried out by the GT-C superfamily of glycosyltransferases that require polyprenylphosphate-based mannose (PPM) as a sugar donor. The essentiality of lipoglycan synthesis for growth makes the glycosyltransferase that synthesizes PPM, a potential drug target in *Mycobacterium tuberculosis*, the causative agent of tuberculosis. In *M. tuberculosis*, PPM has been shown to be synthesized by Ppm1 in enzymatic assays. However, genetic evidence for its essentiality and *in vivo* role in LM/LAM and PPM biosynthesis is lacking. In this study, we demonstrate that *MSMEG3859*, a *Mycobacterium smegmatis* gene encoding the homologue of the catalytic domain of *M. tuberculosis* Ppm1, is essential for survival. Depletion of *MSMEG3859* in a conditional mutant of *M. smegmatis* resulted in the loss of higher order phosphatidylmyo-inositol mannosides (PIMs) and lipomannan. We were also able to demonstrate that two other *M. tuberculosis* genes encoding glycosyltransferases that either had been shown to possess PPM synthase activity (*Rv3779*), or were involved in synthesizing similar polyprenol-linked donors (*ppg5*), were unable to compensate for the loss of *MSMEG3859* in the conditional mutant.

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Introduction

Tuberculosis (TB) affects a third of mankind and causes 1.7 million fatalities annually [1]. The spread of TB has been facilitated in recent decades due to the susceptibility of HIV-infected individuals to *Mycobacterium tuberculosis*, the etiological agent of TB [2]. The problem has been compounded by the emergence of multi- and extensively-drug resistant *M. tuberculosis* strains [2]. Typically, the cell walls of the genus *Mycobacterium* contain mycolic acids (m), arabinogalactan (AG) and peptidoglycan (P), which are covalently linked to each other to form the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex [3]. A particular group of specialized glycopospholipids, phosphatidylmyo-inositol (PI) mannosides (PIMs) and lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), are found in the outer leaflet of mAGP [4]. LM and LAM, which are based on a core PIM unit, possess an elongated $\alpha(1\rightarrow6)$ linear, $\alpha(1\rightarrow2)$ branched mannan, of approximately 30 mannopyranose (Man β) residues, and linked to its terminus to a branched D-arabinan domain of approximately 70 arabinofuranose (Ara β) residues, assembled through several distinct structural motifs [4–6]. In mycobacteria, the large arabinan domain is capped to various degrees with short $\alpha(1\rightarrow2)$ Man β chains in the case of *M. tuberculosis* [7], whereas in *M. smegmatis*, caps of inositol phosphate are present and termed PILAM [4], and *M. chelonae* possess non-capped LAM [8]. Both

ManLAM and PILAM exhibit a broad range of immunomodulatory activities. For example ManLAM, which is predominantly found in the slow-growing pathogenic mycobacteria, inhibits a number of immune system effector functions, including interferon- γ -mediated activation of macrophages [4]. ManLAM also inhibits the production of the pro-inflammatory cytokines interleukin-12 [4] and tumor necrosis factor- α [9]. PILAM, which is characteristic of the fast-growing saprophytic mycobacteria, can induce a pro-inflammatory response in a Toll-like 2 receptor-dependent manner [10].

The current model of lipoglycan biosynthesis is supported by biochemical and genetic studies, and follows a linear pathway from PI \rightarrow PIM $_2$ \rightarrow LM \rightarrow LAM [4]. PimA (Rv2610c) and PimB' (Rv2188), are α -mannopyranosyltransferases, belonging to the GT-B superfamily and utilize GDP-mannose [11,12], adding Man β residues at positions O-2 and O-6 of PI, respectively to produce PIM $_2$, that can be subsequently acylated by Rv2611c, at the 6 position of the Man β residue to generate Ac $_1$ /Ac $_2$ PIM $_2$ [13]. RvD2-ORF1 from *M. tuberculosis* CDC1551, designated as PimC and an unknown glycosyltransferase (PimD), catalyzes the addition of a Man β residue from GDP-mannose to 6-OH of mannose at the non-reducing end of Ac $_1$ /Ac $_2$ PIM $_2$ to generate Ac $_1$ /Ac $_2$ PIM $_3$ [14] and Ac $_1$ /Ac $_2$ PIM $_4$, respectively. At this key junction point in the biosynthetic pathway, polyprenylphosphate-based mannose (PPM) donors are employed by the GT-C superfamily for elongation and

branching of Ac₁/Ac₂PIM₄ leading to Ac₁/Ac₂PIM₆, LM and LAM [15].

The pathway splits into two branches from Ac₁/Ac₂PIM₄: one leads to the formation of Ac₁/Ac₂PIM₆, whilst the other leads to the formation of LM and LAM. In the first branch, $\alpha(1\rightarrow2)$ Man β is added to Ac₁/Ac₂PIM₄ to form Ac₁/Ac₂PIM₆ by two consecutive mannose additions catalyzed by PimE (Rv1159), a PPM-dependent $\alpha(1\rightarrow2)$ -mannopyranosyltransferase and a second additional uncharacterized putative glycosyltransferase of the GT-C superfamily [16]. In the second branch, Ac₁/Ac₂PIM₄ is hypermannosylated by glycosyltransferases of the GT-C superfamily to generate LM. Recent studies have established MptB (Rv1459c) and MptA (Rv2174) to be involved in the synthesis of the mannan backbone, where MptB catalyzes the synthesis of the proximal end through the addition of 12–15 Man β residues to the backbone and MptA synthesizes the distal end of the $\alpha(1\rightarrow6)$ mannan core of LM [17–19]. The $\alpha(1\rightarrow6)$ -mannan core, synthesized by MptB and MptA, is further branched by MptC (Rv2181) which adds $\alpha(1\rightarrow2)$ -Man β residues to the side chains of LM [20]. The transition from LM to LAM is catalyzed by EmbC (Rv3793) and involves the utilization of LM through initial priming with Araf units from the sugar donor decaprenylphosphate arabinan (DPA), by an unknown GT-C glycosyltransferase [21]. EmbC is then responsible for extension of the primed LM through the addition of 12–16 $\alpha(1\rightarrow5)$ -Araf residues [22]. The addition of branch points is similar to AG, catalyzed by AftC [23]. The arabinan domain as found for AG synthesis is likely to be terminated by AftB (Rv3805c) [24]. The homologue of Rv1635c in *M. tuberculosis* CDC1551 has been shown to be involved in Man-LAM capping [6]. This enzyme was also shown to be PPM-dependent and is now termed as CapA, which adds the first Man β residue onto the non-reducing arabinan termini of LAM [25]. In *M. tuberculosis*, MptC (Rv2181) a PPM-dependent $\alpha(1\rightarrow2)$ mannosyltransferase has dual functionality in branching LM, and also for producing ManLAM that is fully functional *via* the capping of LAM with $\alpha(1\rightarrow2)$ -Man β residues at the non-reducing end of LAM [4].

In vitro studies have shown that the key PPM sugar donor for the GT-C glycosyltransferases involved in LM/LAM biosynthesis is generated by Mt-Ppm1 (Rv2051c), a DPM-like synthase that uses GDP-Man and C₅₀/C₃₅-polyprenol phosphates as substrates [26]. Previous studies have identified subtle variations in the organization of the *ppm1* locus in mycobacteria [26]. The well characterized *Mt-ppm1* of *M. tuberculosis* encodes a large polypeptide consisting of two domains, *Mt-ppm1/D1*, which is membrane-anchored *via* six transmembrane segments, and *Mt-ppm1/D2* which is sufficient for DPM synthase activity [26,27]. However, in *Mycobacterium smegmatis*, these two domains are encoded by two distinct ORFs *MSMEG3860* (Domain 1, *Ms-ppm2*) and *MSMEG3859* (Domain 2, *Ms-ppm1*) arranged in an operon (Figure 1), an arrangement also found in *Mycobacterium leprae*, *Mycobacterium avium* and the related *Corynebacterium glutamicum* [26,28]. Studies using bacterial two-hybrid systems have shown that *MSMEG3859* and *MSMEG3860* interact with each other [27]; while *MSMEG3859* was sufficient for PPM synthase activity, this interaction with *MSMEG3860* stabilized the synthase [26,27]. Similarly, in *M. tuberculosis*, a recombinant protein consisting only of the C-terminal domain (*Mt-Ppm1/D2*) was sufficient for generation of PPM activity [26]. However, whilst *in vitro* data has shown that Mt-Ppm1 (and *MSMEG3859*) has PPM synthase activity, there have been no genetic studies that demonstrate that Mt-Ppm1 is the sole PPM generating enzyme encoded by the *M. tuberculosis* genome. If this was the case, in view of the critical role of PPM for biosynthesis of LM/

LAM, *Mt-ppm1* would be expected to be an essential gene and thus genetic studies would be possible only via the generation of a conditional knockout strain. The need to study the *in vivo* role of *ppm1* by generation of a conditional mutant strain was further necessitated by conflicting reports that another membrane-associated glycosyltransferase, Rv3779 functions as a PPM synthase in *M. tuberculosis* [29], and as a glycosyltransferase that uses polyprenyl-P-D-GalNAc as a D-GalN β (or D-GalNAc) donor for transfer to 3,5-branched D-Araf residues of AG [30]. Additionally, the latter report also describes *Rv3631* (*ppgS*) and *Rv3632* as genes encoding a polyprenyl-P-D-GalN synthase and a small integral membrane protein respectively, analogous to Mt-Ppm1/D2 and Mt-Ppm1/D1. While *PpgS* is a GT-2 family glycosyltransferase involved in the generation of polyprenylphospho-*N*-acetylgalactosamine (polyprenyl-P-GalNAc) from polyprenyl-P and UDP-GalNAc, it could be envisaged as a secondary, potential PPM synthase.

In an effort to first confirm the *in vivo* role of Mt-Ppm1/D2 in LM/LAM biosynthesis we aimed to test the essentiality of PPM glycosyltransferase activity in *M. smegmatis* by using CESTET, a genetic tool for testing gene essentiality in *M. smegmatis*. As *M. smegmatis* does not encode homologues of *ppgS* or *Rv3779*, it also provided us with a valuable surrogate to probe the *in vivo* role and potential essentiality of *MSMEG3859* in the absence of any potential functional redundancy caused by an alternative PPM synthase. Additionally, mutant or conditional mutant strains of *M. smegmatis* could subsequently be used as a host strain to test functional complementation of PPM synthase activity using recombinant *ppgS* or *Rv3779*.

Materials and Methods

Construction of Recombinant Plasmids

For generating an integrative vector containing *MSMEG3859*, the ORF was amplified from *M. smegmatis* mc²155 [31] genomic DNA using the primers F3859 (5'-TCGGAATTCATGAGCGTCCCCGGTGAACG-3') and R3859 (5'-GCTATCGATTACAGCGGACCACGCCCTGG-3'), cloned downstream of the tetracycline promoter in the integrative vector pTIC6a vector (gift from A. Baughn and W.R. Jacobs Jr., Albert Einstein College of Medicine, NY) and named pTIC6a-*MSMEG3859*. For subsequent complementation/rescue experiments, the plasmid pMV261Apra, a derivative of pMV261 [32], was used for cloning various GTFs. *MSMEG3859* was amplified using primer pairs F3859A (5'-GCGGTCAGCTGATGAGCGTCCCCG-3') and R3859 (5'-GCTATCGATTACGCCACCACGCCCTGG-3'); the C-terminal domain of *Rv2051c* (*Mt-ppm1/D2*) using primer pairs F2051C (5'-GCTGCTGGCCAACCACCGGCCAGC-3') and R2051C (5'-TCGGAATTCACCACCGGCCAGCCG-3'); *Rv3779* using F3779 (5'-GATGGC-CAGTGGGCTGTGGTTTCG-3') and R3779 (5'-ATAAGCTTCC TAGGAGTGTGTTGC-3'); *Rv3631* (*ppgS*) using F3631 (5'-GCTCGTGGCCAA TGGCCTCGAAAA-3') and R3631 (5'-TCGACATCGATTACGTCGTGGC-3'). The-PCR amplified DNA fragments of *MSMEG3859*, *Mt-ppm1/D2*, *Rv3779* and *ppgS* were cloned into pMV261Apra using primer-incorporated restriction sites and the resultant plasmids were named pAKR-*MSMEG3859*, pAKR-*Mt-ppm1/D2*, pAKR-*Rv3779* and pAKR-*ppgS*, respectively.

Generation of the Δ MsPpm Conditional Mutant

The *M. smegmatis* conditional mutant Δ MsPpm was generated using CESTET [33]. Briefly, a merodiploid was first generated by introducing pTIC6a-*MSMEG3859* by electroporation into

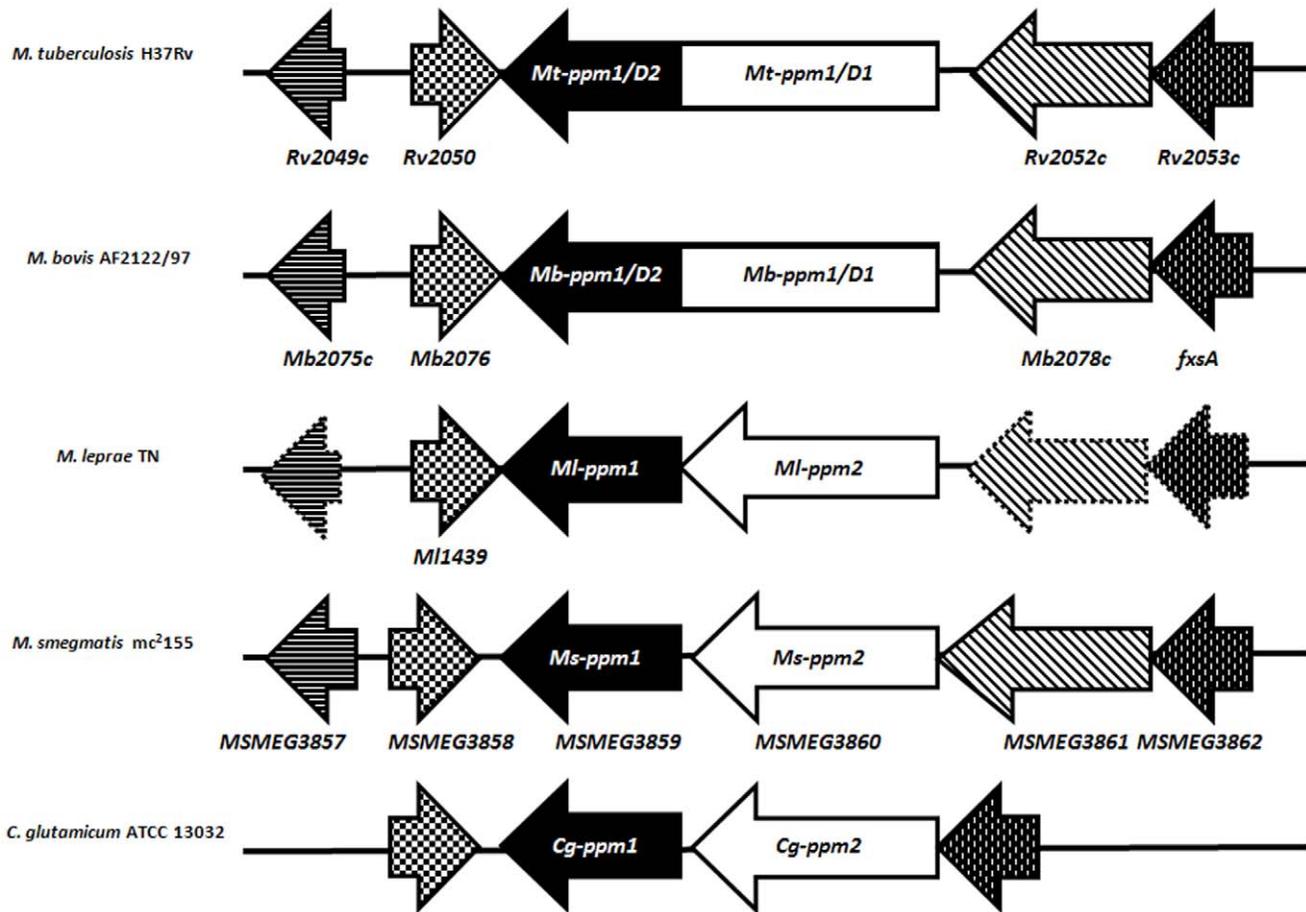


Figure 1. Genomic organization of *ppm1* region in different mycobacteria and in *Corynebacterium glutamicum*. Homologous genes are indicated by similar arrows and pseudogenes in *M. leprae* are indicated by arrows with dotted borders. In *M. tuberculosis* and *M. bovis*, the *ppm1*-encoded protein consist of two domains fused together, while these two domains are encoded by two distinct ORFs in *M. leprae*, *M. smegmatis* and *C. glutamicum*.

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M. smegmatis mc²155 [33]. The merodiploid strain mc²155::pTIC6a-MSMEG3859 was then subjected to specialized transduction as previously described [34] using a temperature-sensitive, recombinant phage Δ MsPpm designed to replace MSMEG3859-MSMEG3860 with a hygromycin resistance marker. Transductants were selected at the non-permissive temperature of 37°C on selective plates containing 25 μ g/ml kanamycin, 100 μ g/ml hygromycin B and 50 ng/ml anhydrotetracycline (ATc). After confirmation of gene replacement by Southern blot, one such transductant was named Δ MsPpm and was selected for further analysis.

Conditional Depletion of Δ MsPpm Conditional Mutant

The Δ MsPpm conditional mutant was grown in Tryptic Soy Broth (TSB; Difco) containing 0.05% Tween 80, 25 μ g/ml kanamycin, 100 μ g/ml hygromycin B and 50 ng/ml ATc and subsequently passaged twice in medium without ATc. To visualize the effects of the conditional depletion of MSMEG3859 in Δ MsPpm on lipids and lipoglycans, the strains were grown to OD 0.8, labelled with 10 μ Ci/ml glucose D-[¹⁴C(U)] (specific activity 250–360 mCi (9.25–13.3 GBq)/mmol; Perkin Elmer) and incubated at 37°C for 4 hours.

Extraction of Polar Lipids

Polar lipids and apolar lipids were extracted as described previously [35]. Briefly, cells from a 10 ml volume culture were washed once with 2 ml phosphate buffer saline (PBS) and treated with 2 ml CH₃OH:0.3% NaCl_(aq) (100:10, v/v) and 2 ml petroleum ether for 30 min. The suspension was centrifuged and the upper layer containing apolar lipids was separated. An additional 2 ml of petroleum ether was added, mixed and centrifuged as described above and the two upper apolar lipid fractions were combined and dried. For polar lipids, 2.3 ml of CHCl₃:CH₃OH:0.3% NaCl_(aq) (90:100:30, v/v/v) was added to the cell pellet and mixed for 1 h. This mixture was centrifuged and the supernatant was separated. The remaining cell pellet was mixed with 750 μ l of CHCl₃:CH₃OH:0.3% NaCl_(aq) (50:100:40, v/v/v) for 30 min, centrifuged and the supernatant was combined to the previous fraction. After repeating this step, 1.3 ml of CHCl₃ and 1.3 ml of 0.3% NaCl_(aq) was mixed with the pooled supernatant, centrifuged, and the lower layer containing the polar lipids was recovered and dried. The polar lipid extracts were dried and resuspended in CHCl₃:CH₃OH (2:1, v/v), and incorporation of glucose D-[¹⁴C(U)] was quantified by liquid scintillation counting using 5% of the lipid fractions in 5 ml EcoScint A (National Diagnostics). Equal counts of polar lipid extracts (50 000 cpm) were applied to Silica Gel 60 F₂₅₄ (Merck 5554)

aluminium-backed TLC plates and developed using solvent system E for polar lipids: $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (60:30:6, v/v/v) in the first direction and $\text{CHCl}_3:\text{CH}_3\text{CO}_2\text{H}:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film).

Extraction and Purification of Lipoglycans

Lipoglycans were extracted as described previously [36]. Briefly, dried cells from a 10 ml volume culture were resuspended in water and refluxed five times with equal volume of 50% $\text{C}_2\text{H}_5\text{OH}$ at 85°C , for 6 h intervals, followed by centrifugation and recovery of the supernatant. The combined supernatants were dried and subjected to hot phenol- H_2O treatment at 65°C . The aqueous phase containing the crude lipoglycan fraction was dialyzed against water, dried and the incorporation of glucose D- $^{14}\text{C}(\text{U})$ was quantified by liquid scintillation counting using 5% of the lipoglycan fractions in 5 ml EcoScint A (National Diagnostics). Equal counts (50 000 cpm) were loaded on a 15% SDS-PAGE gel and separated by electrophoresis. Lipoglycans were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film).

PPM Synthase Assay

Membranes were prepared as described previously [37]. Briefly, cells were grown to mid-log phase, harvested, washed with PBS and stored at -20°C . Cells were washed and resuspended in buffer A containing 50 mM MOPS (adjusted to pH 7.9 with KOH), 5 mM β -mercaptoethanol and 10 mM $\text{MgCl}_{2(\text{aq})}$ at 4°C and subjected to sonication for a total time of 10 min using 60 s pulses and 90 s cooling intervals. The preparations were centrifuged at $27000\times g$ for 25 min at 4°C and the membranes were obtained by further centrifugation of the supernatant at $100,000\times g$ for 1 h at 4°C . Membranes were resuspended in 1 ml of buffer A and concentration was determined using the BCA Protein Assay Reagent Kit (Thermo Scientific). Reaction mixtures for assessing $^{14}\text{C}[\text{Man}]$ incorporation consisted of 6.25 μCi $\text{GDP}[\text{Man-}^{14}\text{C}(\text{U})]$ (262 mCi/mmol; Perkin Elmer), 100 μM ATP, 10 mM $\text{MgCl}_{2(\text{aq})}$, 100 μM dithiothreitol, 20 mM $\text{NaF}_{(\text{aq})}$ and membrane preparations corresponding to 50–400 μg protein in a final volume of 100 μl . Decaprenyl monophosphate was added to the reaction mixtures at a final concentration of 125 μM . The reaction mixtures were then incubated at 37°C for 30 min. The enzymic reactions were terminated by the addition $\text{CHCl}_3/\text{CH}_3\text{OH}/0.8\text{ M NaOH}_{(\text{aq})}$ (10:10:3 by vol.) (6 ml/100 μl) followed by further incubation at 55°C for 20 min. The mixtures were then allowed to cool; 2.625 ml of CHCl_3 and 1.125 ml of water were added. The mixture was vortexed and centrifuged and the upper aqueous phase discarded. The organic phase was washed three times with 2 ml of $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (3:47:48 by vol.), dried to yield an organic fraction containing PPMs. These were dried in a scintillation vial before scintillation counting using 5 ml of EcoScint A (National Diagnostics) [26].

Results and Discussion

Essentiality of MSMEG3859 in *M. smegmatis*

Due to its role in the biosynthesis of LM/LAM, *Mt-ppm1* was considered to be an essential gene. We constructed a knockout phage $\text{ph}\Delta\text{MsPpm}$ designed to replace *MSMEG3859-MSMEG3860* in *M. smegmatis* with a hygromycin resistance cassette; we were however unable to generate a null mutant due to the failure to yield any transductants. In contrast, we were able to generate a *MSMEG3859-MSMEG3860* null mutant by trans-

ducing a merodiploid strain containing a second, inducible copy of *MSMEG3859-MSMEG3860* by CESTET (Conditional Expression Specialized Transduction Essentiality Test) [33]; suggesting that one or both genes were essential in *M. smegmatis*. As *MSMEG3859* was shown to be sufficient for the enzymatic generation of PPM *in vitro*, we reasoned that the PPM synthase-encoding *MSMEG3859*, but not the membrane segment-encoding *MSMEG3860*, was an essential gene. To address this, we used CESTET again to test the essentiality of *MSMEG3859* in *M. smegmatis*. First, a merodiploid strain was constructed by introducing a second copy of *MSMEG3859* cloned in pTIC6a, an integrative plasmid driving expression *via* a tetracycline inducible promoter [38]. Expression of this recombinant copy of *MSMEG3859* can be induced by adding anhydrotetracycline (ATc) to the growth medium. Following transduction with knockout phage $\text{ph}\Delta\text{MsPpm}$, we were able to generate knockout mutants only when transductants were selected on plates containing hygromycin and ATc suggesting that *MSMEG3859* was essential in *M. smegmatis*. One such conditional mutant, designated ΔMsPpm was used for further analysis. Subsequent passages of the ΔMsPpm mutant in medium without the inducer ATc resulted in loss of viability indicating that expression of the pTIC6a-driven copy of *MSMEG3859* was required for cell growth, confirming the essentiality of *MSMEG3859* (Figure 2).

The ability to generate an *MSMEG3859-MSMEG3860* double mutant in a *MSMEG3859* merodiploid strain indicated that while the PPM synthase-encoding *MSMEG3859* was essential, *MSMEG3860* (which encodes a six transmembrane section-containing membrane-anchored protein) was not essential. This

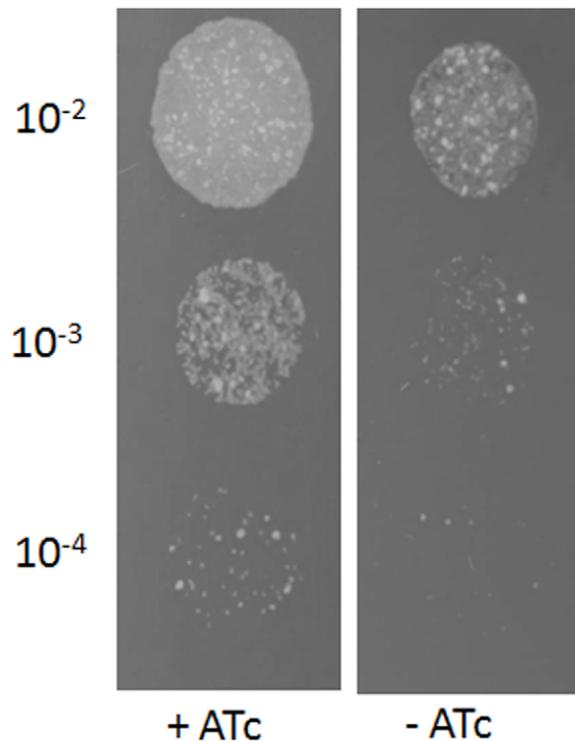


Figure 2. Essentiality of *MSMEG3859* in *M. smegmatis* mc^2155 . Growth of the ΔMsPpm conditional mutant on Tryptic Soy Agar with or without the inducer anhydrotetracycline (ATc). Ten microliters of 10-fold serial dilutions of cultures were spotted on the agar plates and incubated for 3 days at 37°C . doi:10.1371/journal.pone.0048211.g002

correlated with earlier reports that *MSMEG3859* was sufficient for PPM synthase activity [26,27].

Loss of *MSMEG3859* Results in Alteration of PIMs and Cessation of LM Biosynthesis

As Ac_1/Ac_2PIM_4 is at the branch point for the biosynthesis of higher PIMs (Ac_1/Ac_2PIM_6) and LM/LAM biosynthesis, the conditional $\Delta MsPpm$ mutant could be used to determine whether loss of *MSMEG3859*-encoded PPM activity affected the biosynthesis of these molecules. The mutant was grown for 36 hours in media in the presence, or absence, of ATc labelled with [^{14}C]-glucose and subjected to lipid extractions. Cultures grown in ATc-containing media showed all PIM intermediates present. In contrast, cultures of the conditional mutant grown in the absence of ATc showed increasing amounts of PI accompanied by decreasing levels of Ac_1/Ac_2PIM_6 (Figure 3). The loss of Ac_1/Ac_2PIM_6 and accumulation of PIM_x precursors suggested that *MSMEG3859* is required for the synthesis of higher order PIMs, particularly Ac_1/Ac_2PIM_6 . Conditional depletion of PPM synthase activity in the $\Delta MsPpm$ mutant should also affect the biosynthesis of lipoglycans and cultures grown in the absence of ATc did show diminished levels of [^{14}C]LM (Figure 4). However, we did not see major differences in [^{14}C]LAM levels. The loss of [^{14}C]LM but presence of [^{14}C]LAM in the depleted cultures could be explained by the residual LM molecules in the depleted cells being extended to form [^{14}C]LAM from endogenous PPM generated prior to conditional depletion. Thus, the observed LAM in the depleted cells was likely from residual LM, rather than newly synthesised LM, as observed through dual [^{14}C]/[3H] dual labelling experiments by Besra et al. [37]. These data suggest that *MSMEG3859*, which encodes the equivalent of *M. tuberculosis* Ppm1/D2, is solely responsible for the PPM synthase activity required for the generation of higher order PIMs and LM/LAM.

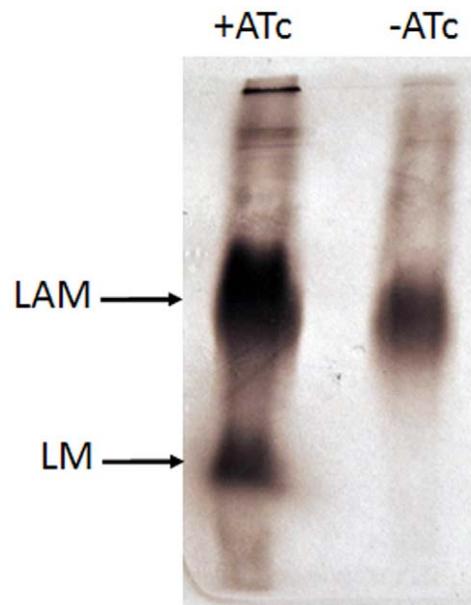


Figure 4. Lipoglycan profile of the $\Delta MsPpm$ conditional mutant. [^{14}C]-labelled lipoglycan fractions were separated on 15% SDS-PAGE gel from cultures of the $\Delta MsPpm$ conditional mutant with or without anhydrotetracycline (ATc). Equal counts (50 000 cpm) were loaded on a 15% SDS-PAGE. Lipoglycans were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film). doi:10.1371/journal.pone.0048211.g004

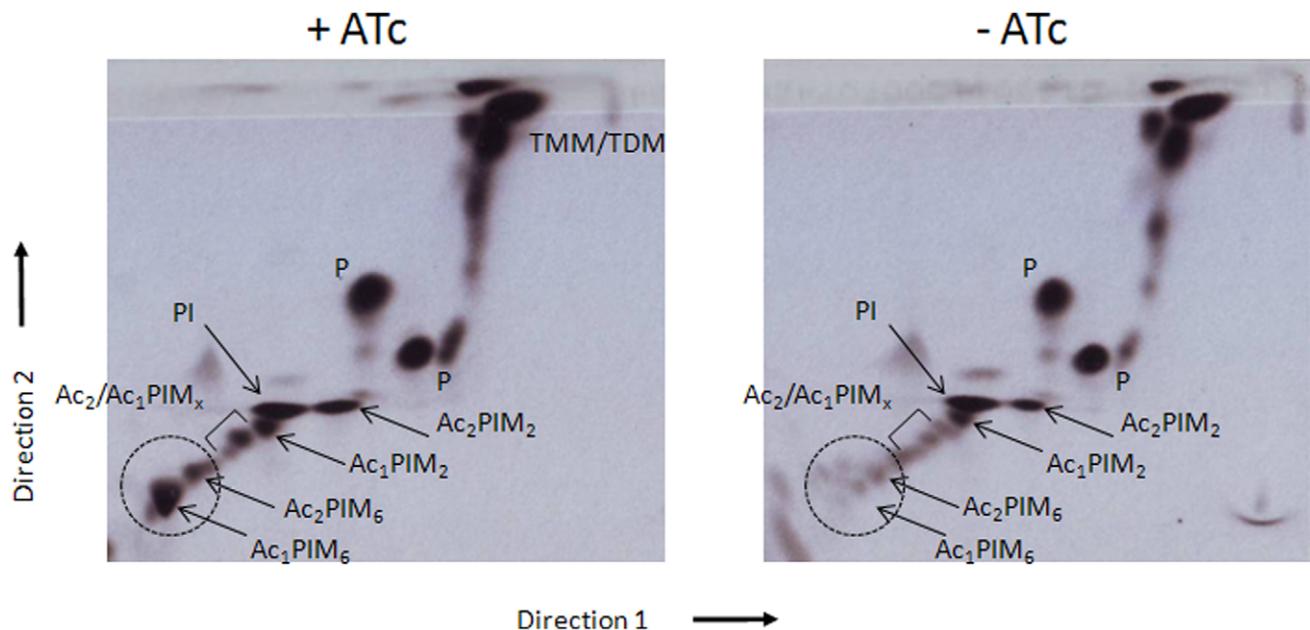


Figure 3. 2D-TLC analysis of [^{14}C]-labeled polar lipids from the $\Delta MsPpm$ conditional mutant. Cultures were grown and labelled in Tryptic Soy Broth in the presence or the absence of anhydrotetracycline (ATc). Equal counts of polar lipid extracts (50 000 cpm) were applied to Silica Gel 60 F₂₅₄ (Merck 5554) aluminium-backed TLC plates and developed using solvent system E for polar lipids: $CHCl_3/CH_3OH/H_2O$ (60:30:6, v/v/v) in the first direction and $CHCl_3/CH_3OOH:CH_3OH:H_2O$ (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film). PIM intermediates are shown by arrows ($x = 3-5$) and the dotted circle indicates the position of the higher PIMs on the TLC plates. doi:10.1371/journal.pone.0048211.g003

Effects of Loss of MSMEG3859 on Membrane-associated PPM Synthase Activity

Membrane preparations from cultures of the Δ MsPpm mutant, grown in the presence and in the absence of ATc, were used to assay membrane-associated PPM synthase activity. A PPM synthase assay was used to analyse PPM activity in the membranes using GDP-[14 C] Man and polyprenol phosphate as substrate. This assay was used to assess the effects of depletion of MSMEG3859 function on the ability of the membrane preparations to catalyse the formation of PPM (C₅₀-P-Man) donors *via* the incorporation of radioactive mannose into polyprenol substrate through pooled organic extracts containing PPMs [26]. While membrane preparations from cultures grown in the presence of ATc were able to catalyse incorporation of [14 C] Man into polyprenols, those from cultures grown in the absence of ATc displayed poor PPM synthase activity (Figure 5). This co-relation between depleted MSMEG3859 function and low PPM synthase activity confirmed that MSMEG3859 was the key synthase required to catalyze the production of PPM donors in *M. smegmatis*.

Together, the data obtained from the depletion experiments with the conditional mutant highlight the potential of the MSMEG3859-encoded PPM synthase as a potential drug target that affects not only viability, but also the biosynthesis of an immunomodulatory mycobacterial lipoglycan.

Potential Ability of the Alternative *M. tuberculosis* PPM Synthases to Rescue Viability and Restore Wild-type Phenotype in the Δ MsPpm Mutant

In contrast to *M. smegmatis*, *M. tuberculosis* encodes two other membrane-associated glycosyltransferases, Rv3779 and Rv3631 (PpgS), which have been suggested to have putative roles as PPM synthases in *M. tuberculosis* [30]. As mentioned above, homologues of neither are not present in *M. smegmatis*. The Δ MsPpm conditional mutant thus offered us an opportunity to assess the possible roles of Rv3779 and PpgS as alternative PPM synthases.

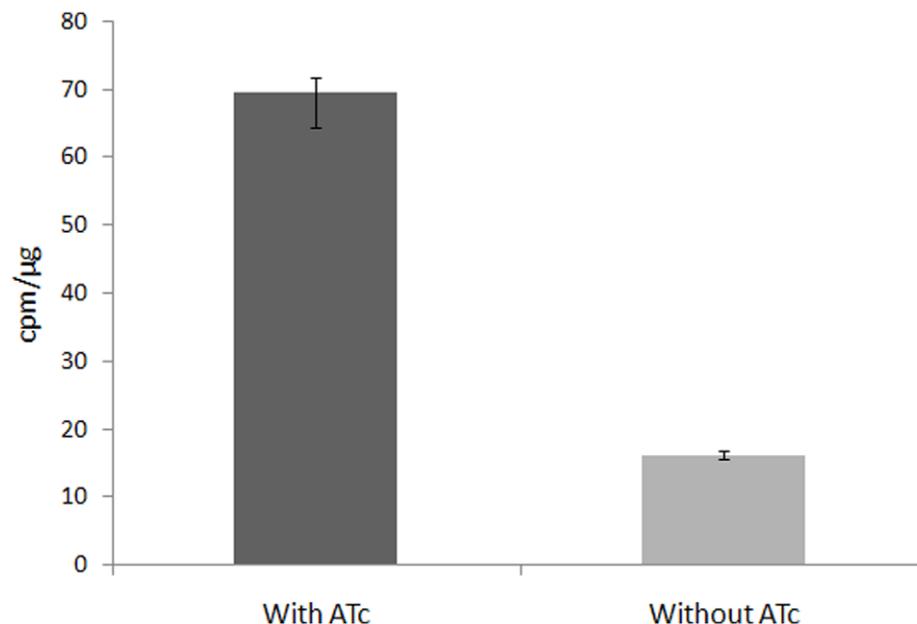


Figure 5. PPM synthase activity in membranes of the Δ MsPpm conditional mutant. Graph shows the transfer of [14 C]-mannose from GDP[Man- 14 C(U)] (262 mCi/mmol; Perkin Elmer) to polyprenol-phosphate in membrane extracts prepared from cultures grown with or without anhydrotetracycline (ATc). doi:10.1371/journal.pone.0048211.g005

Each gene could be functionally analyzed *in vivo* by testing the ability of the Δ MsPpm conditional mutant transformed with a plasmid-borne copy of either Rv3779 or ppgS to rescue Ac₁/Ac₂PIM₆ biosynthesis when cultured in medium devoid of ATc. As expected, Ac₁/Ac₂PIM₆ biosynthesis was not affected in non-ATc cultures of Δ MsPpm containing plasmid clones of either MSMEG3859 (Figure 6). We then tested the ability of the *M. tuberculosis* equivalent, Mt-ppm1/D2 to rescue the loss of MSMEG3859 function in the same manner and found Ac₁/Ac₂PIM₆ levels unaffected in the recombinant strains (Figure 6) indicating that Mt-ppm1/D2 was functional in *M. smegmatis*. In contrast, however, Rv3779 was unable to rescue Ac₁/Ac₂PIM₆ biosynthesis in the conditional mutant when grown in the absence of ATc, leading to a loss of Ac₁/Ac₂PIM₆ and a noticeable accumulation of intermediary Ac₁/Ac₂PIM_s (Figure 6). Thus while Rv3779 was shown to have PPM synthase activity *in vitro* [29], it surprisingly failed to substitute for loss of MSMEG3859 in the conditional Δ MsPpm mutant. Thus, in contrast to its *in vitro* activity, it is unlikely that Rv3779 functions *in vivo* as a PPM synthase. Instead, its predominant role in *M. tuberculosis* seems to be the utilisation of polyprenyl-P-D-GalNAc as a donor for the biosynthesis of galactosamine-modified AG [30].

As mentioned above, while PpgS is involved in the generation of polyprenyl-phospho-N-acetylgalactosamine (polyprenyl-P-GalNAc) from polyprenyl-P and UDP-GalNAc, it could be envisaged as a secondary, potential PPM synthase. Its syntenic association with the small integral membrane protein Rv3662 is analogous to MSMEG3859-MSMEG3860, and Mt-Ppm1/D2-Mt-Ppm1/D1. However, ppgS also failed to compensate for the loss of MSMEG3859 in the conditional mutant (Figure 6), and thus is unlikely to function as a PPM synthase *in vivo*.

Together, these results indicate that ppm1 is the gene encoding the sole PPM synthase in *M. tuberculosis* capable of generating the mannose donor for subsequent higher order PIMs and LM/LAM biosynthesis.

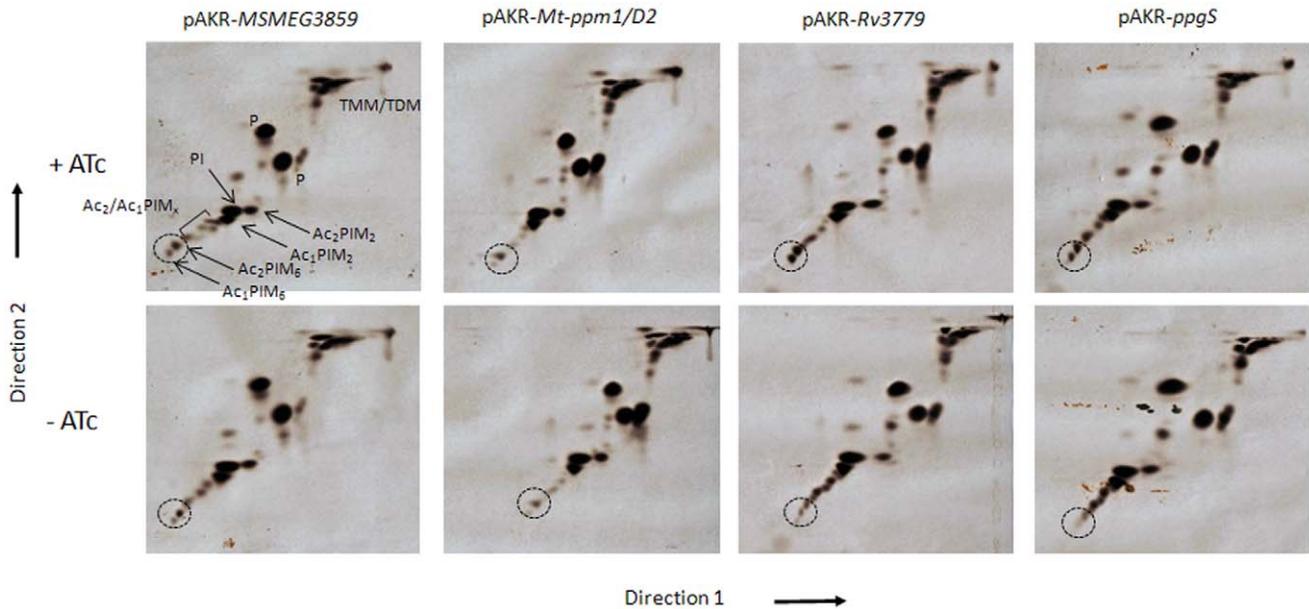


Figure 6. Complementation of the Δ MsPpm conditional mutant. 2D-TLC analysis of [14 C]-labelled polar lipids from the Δ MsPpm conditional mutant complemented with (A) pAKR- *MSMEG3859* (B) pAKR-*Mt-ppm1/D2* (C) pAKR-*Rv3779* (D) pAKR-*ppgS*. Equal counts of polar lipid extracts (50 000 cpm) were applied to Silica Gel 60 F₂₅₄ (Merck 5554) aluminium-backed TLC plates and developed using solvent system E for polar lipids: CHCl₃/CH₃OH/H₂O (60:30:6, v/v/v) in the first direction and CHCl₃:CH₃OOH: CH₃OH:H₂O (40:25:3:6, v/v/v/v) in the second direction. Polar lipids were visualized by 48 h exposure on x-ray films by autoradiography (Kodak Biomax MR film). PIM intermediates are shown by arrows (x=3–5) and the dotted circle indicates the position of the higher PIMs on the TLC plates.
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Author Contributions

Conceived and designed the experiments: AB LRC GSB. Performed the experiments: AR AS SG. Analyzed the data: AR SG LRC AB GSB.

Contributed reagents/materials/analysis tools: AB GSB. Wrote the paper: AR LRC AB GSB.

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