New Insights into the Early Steps of Phosphatidylinositol Mannoside Biosynthesis in Mycobacteria

PimB is an Essential Enzyme of Mycobacterium Smegmatis

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Phosphatidyl-myoinositol mannolipids (PIMs) are key glycolipids of the mycobacterial cell envelope. They are considered not only essential structural components of the cell but also important molecules implicated in host-pathogen interactions. Although their chemical structures are well established, knowledge of the enzymes and sequential events leading to their biosynthesis is still incomplete. Here we show for the first time that although both mannosyltransferases PimA and PimB (MSMEG_4253) recognize phosphatidyl-myoinositol (PI) as a lipid acceptor, PimA specifically catalyzes the transfer of a Manp residue to the 2-position of the myo-inositol ring of PI, whereas PimB exclusively transfers to the 6-position. Moreover, whereas PimB can catalyze the transfer of a Manp residue onto the PI-monomannoside (PIM1) product of PimA, PimA is unable in vitro to transfer Manp onto the PIM1 product of PimB. Further assays using membranes from Mycobacterium smegmatis and purified PimA and PimB indicated that the acylation of the Manp residue transferred by PimA preferentially occurs after the second Manp residue has been added by PimB. Importantly, genetic evidence is provided that pimB is an essential gene of M. smegmatis. Altogether, our results support a model wherein Ac1PIM2, a major form of PIMs produced by M. smegmatis, arises from the consecutive action of PimA, followed by PimB, and finally the acyltransferase MSMEG_2934. The essentiality of these three enzymes emphasizes the interest of novel anti-tuberculosis drugs targeting the initial steps of PIM biosynthesis.

PIMs are unique mannolipids found in abundant quantities in the inner and outer membranes of the cell envelope of Mycobacterium spp. and a few other actinomyces. They are based on a phosphatidyl-myoinositol (PI) lipid anchor carrying one to six Manp residues and up to four acyl chains (for review see Refs. 1, 2). Based on a conserved mannosyl-PI anchor, they are also thought to be the precursors of the two major mycobacterial lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM) (1, 2). PIMs, LM, and LAM are considered not only essential structural components of the mycobacterial cell envelope (3–6), but also important molecules implicated in host-pathogen interactions in the course of tuberculosis and leprosy (1).

Although the chemical structure of PIMs is now well established, knowledge of the enzymes and sequential events leading to their biosynthesis is still fragmentary. According to the currently accepted model, the biosynthetic pathway is initiated by the transfer of two Manp residues and a fatty acyl chain to PI in the cytoplasmic leaflet of the plasma membrane. Based on genetic and biochemical evidence, Kordulakova et al. (5) identified PimA (MSMEG_2935 in Mycobacterium smegmatis mc2155) as the enzyme that catalyzes the first mannosylation step of the pathway transferring a Manp residue most likely to the 2-position of the myo-inositol (myo-Ins) ring of PI. In contrast, the identity of PimB, the enzyme responsible for the transfer of the second Manp to the 6-position of the myo-Ins ring of PIM1, remains controversial. The Rv0557 protein of Mycobacterium tuberculosis H37Rv (PimB; MSMEG_1113 in M. smegmatis mc2155) was originally characterized as PimB (7). However, the lack of an Rv0557 ortholog in the genome of Mycobacterium leprae and the fact that the disruption of this gene in M. tuberculosis Erdman did not significantly affect the biosynthesis of PIMs suggest that compensatory activities exist in the bacterium or that Rv0557 serves another primary function (8, 9). Somewhat supporting the latter hypothesis, the ortholog of Rv0557 in Corynebacterium glutamicum (NCg0452, renamed mgtA) was implicated in the mannosylation of a novel glycolipid (1,2-di-O-C16/C18:1-(α-D-mannosyl)-(1→4)-(α-D-glucopyranosyluronic acid)-(1→3)-glycerol), and Rv0557 from M. tuberculosis was reported to functionally complement for this enzyme in a C. glutamicum knock-out mutant (10). However, to our knowledge this mannosylated glycolipid has never been reported in mycobacteria, and it remains unclear whether PimB serves a similar physiological function in Mycobacterium spp.
More recently, Lea-Smith et al. (11) have shown that the biosynthesis of Ac1PIM2, Ac2PIM2, from Ac1PIM1 in *C. glutamicum* is catalyzed by NCgl2106 (Cg-PimB). Disruption of the NCgl2106 gene totally abolished Ac1PIM2 production in the mutant, arguing against the existence of a compensatory activity associated with the Corynebacterial PimB enzyme. Although Ac1PIM2 production in Cg-pimB' and Cg-pimB'/Cg-pimB knock-out mutants was restored upon complementation with the *M. tuberculosis* Rv2188c gene (11, 12), direct evidence that Rv2188c carried out the same physiological function in mycobacteria has been lacking. Moreover, in light of the recent work carried out the same physiological function in mycobacteria and that no other ManT can compensate for a defined from genomic MSMEG_4253 gene (11, 12), direct evidence that Rv2188c enzymes expressed in *C. glutamicum* (12), whether or not pimB and pimB' could compensate for one another in mycobacteria remained open to speculation.

Both PIM1 and PIM2 can be acylated with palmitate at position 6 of the Manp residue transferred by PimA by the acyltransferase MSMEG_2934 (orthologous to Rv2611c from *M. tuberculosis*) and that no other ManT can compensate for a defined from genomic MSMEG_4253 gene (11, 12), direct evidence that Rv2188c enzymes expressed in *C. glutamicum* (12), whether or not pimB and pimB' could compensate for one another in mycobacteria remained open to speculation.

In this work, clear evidence is provided that PimB' (MSMEG_4253 in *M. smegmatis* mc²155) is the α-ManT responsible for the biosynthesis of PIM2 from PIM1 in mycobacteria and that no other ManT can compensate for a deficiency in this enzyme in *M. smegmatis*. Like PimA (5), PimB' is essential to the growth of *M. smegmatis*. Cell-free assays using purified PimA and PimB' and *M. smegmatis* membrane preparations provide new insights into the sequential events leading to the synthesis of the early forms of PIMs in mycobacteria.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of PimB’ from *M. smegmatis* in Escherichia coli—**The *M. smegmatis* pimB’ gene (Ms*pimB’, MSMEG_4253, 72% amino acid identity to Rv2188c) was amplified from genomic *M. smegmatis* mc²155 DNA by standard PCR using oligonucleotide primers pimB’_NdeI_Fwd (5’-GGAA-TTCCATATGACCGGGGTGGTGGTGGTACC-3’) and pimB’_XhoI_Rev (5’-CCGCCTCGAGCGCCCTGAACGCCTGCCG-GTCGG-3’), and Phusion DNA Polymerase (New England Biolabs). The PCR fragment was digested with NdeI and XhoI and ligated to the corresponding restriction sites of pET29a (Novagen) generating pET29a-Ms*pimB’. The recombinant Ms*pimB’ protein (385 residues) has an additional peptide of 43 residues from amino acids (386LEHHHHHHH393) at the C terminus that includes a histidine tag.

**E. coli** BL21(DE3)pLysS cells transformed with pET29a-Ms*pimB’ were grown in 2 X YT medium supplemented with 25 µg ml⁻¹ kanamycin and 34 µg ml⁻¹ chloramphenicol at 37 °C. Ms*pimB’ expression was induced by adding 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (MP Biomedicals). After 4 h at 37 °C, cells were harvested and resuspended in solution A (50 mM Tris-HCl, pH 8.0) containing protease inhibitors (Complete EDTA-free, Roche Applied Science). Cells were disrupted by sonication (five cycles of 1 min), and the suspension was centrifuged for 20 min at 10,000 × g. The supernatant was applied to a HisTrap chelating column (1 ml; GE Healthcare) equilibrated with solution B (50 mM Tris-HCl, pH 8.0, 500 mM NaCl). The column was then washed with solution B until no absorbance at 280 nm was detected. Elution was performed with a linear gradient of 0–500 mM imidazole in solution B at 1 ml min⁻¹. The resulting preparation displayed a single protein band when run on a 10% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue (supplemental Fig. 1S). The purified enzyme concentration was 10 mg ml⁻¹ in solution A containing 20% glycerol and stored at −80 °C until further use in enzyme assays.

**Enzyme Assays—**The enzymatic activity of Ms*pimA and Ms*pimB’ was monitored using a radiometric assay. The reaction mixture contained 0.0625 µCi of GDP-[C¹⁴]Man (specific activity, 305 mCi mmol⁻¹; Amersham Biosciences), 10 µg of PI (Avanti Polar Lipids; liver PI, [M–H]⁻, m/z = 885.53, where the predominant species contains one polyunsaturated C₂₀ and one C₁₈ fatty acyl chain), 50 µg of purified Ms*pimA, Ms*pimB’, or a mix of Ms*pimA and Ms*pimB’ and 50 mM Tris-HCl, pH 7.5, in a final volume of 250 µl. In some assays, membrane preparations from *M. smegmatis* mc²155 (0.5 µg of proteins) served as the source of lipid acceptors. Reactions were incubated for 2 h at 37 °C and stopped with 1.5 ml of CHCl₃/CH₃OH (2:1, by volume). The PIM-containing organic phase was prepared and analyzed by TLC as described by Korduláková et al. (5). Ms*pimA was purified as described previously (14).

For structural analyses, 500 µl cold GDP-Man replaced GDP-[C¹⁴]Man in the assay mixture described above. The reactions were incubated overnight at 37 °C and stopped by adding 1.5 ml of CHCl₃/CH₃OH (2:1, by volume). The radioactive mannolipid products from 15 reactions were isolated by preparative TLC as described (5).

**Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry—**Compounds 1–5 were mixed with an equal volume of matrix (2,5-dihydroxybenzoic acid dissolved in 10 mg ml⁻¹ acetonitrile/water, 50:50, 0.1% trifluoroacetic acid), and the molecular mass was measured in the negative ion mode by MALDI-TOF MS on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA). External calibration was performed using an eight component calibration mixture on a spot adjacent to the sample.

**NMR Analysis—**One-dimensional and two-dimensional NMR experiments were carried out at 25 °C in a Varian Inova 500-MHz NMR spectrometer (Varian Inc., Palo Alto, CA) using an HCN probe head equipped with shielded z-gradient. Samples were dissolved in 0.6 ml of CHCl₃/CD₃OD (2:1, by volume) and spectra acquired using a 5-mm NMR probe. Typical parameters used for one-dimensional ¹H experiments were as follows: sweep width, 5500 Hz; flip angle, 45°; time domain data points, 32,768; number of transients, 32 or 256; and relaxation delay, 1.5 s. For the complete structural analysis of PIM₁, PIM₂, and Ac₁PIM₂, two-dimensional experiments, including gradient-selected correlation spectroscopy, total correlation
spectroscopy, heteronuclear single quantum coherence spectroscopy, and heteronuclear multiple bond correlation spectroscopy were carried out. Parameters used for two-dimensional correlation spectroscopy and total correlation spectroscopy were as follows: sweep width, 5500 Hz in both F2 and F1 dimensions; time domain data points, 2048; number of free induction decays with t1 increment, 512; number of transients, 32 or 256; and relaxation delay, 1.5 s. Parameters used for heteronuclear single quantum coherence and heteronuclear single quantum coherence spectroscopy were as follows: sweep width, 5500 Hz in F2 and 30,188 Hz in F1; time domain data points, 2048; number of free induction decays with t1 increment, 256; number of transients, 32 or 256; and relaxation delay, 1.5 s. The acquired NMR data were processed using the TOPSPIN 2.1 software (Bruker GmbH, Karlsruhe, Germany).

Construction of M. smegmatis MsPimB’ Conditional Mutant—Essentially the same strategy was used to construct a conditional MsPimB’ mutant of M. smegmatis as was used earlier to generate an MsPimA conditional mutant (5). The M. smegmatis MsPimB’ gene (MSMEG_4253) and flanking regions were amplified from genomic M. smegmatis mc^155 DNA by standard PCR strategies using oligonucleotide primers MsPimB’_KO_Apal_fwd (5’-ATAATGGGCCGGCAAAACT-GCGTGACCTGTACG-3’) and MsPimB’_KO_SpeI_rev (5’-ATTATCTAGTGACCTCggGCGCATCGACG-3’), and Phusion DNA polymerase (New England Biolabs). A disrupted allele of MsPimB’, MsPimB’:::km, was constructed by cloning the kanamycin resistance cassette from pUC4K (GE Healthcare) into the AgeI and Stul sites of MsPimB’, generating a 363-bp deletion within the coding sequence of MsPimB’. MsPimB’:::km was then ligated to pQ200xylE to yield pQMsPimB’_XX, the vector used to generate allelic replacement at the MsPimB’ locus (5). The temperature-sensitive pCG76 derivative (15), pCGMsPimB’, was used as the rescue plasmid to carry a functional copy of the MsPimB’ gene in the conditional mutant.

Homology Modeling of MsPimB’—Homology modeling of MsPimB’ was performed with MODELLER 9 Version 4 (16) using the atomic coordinates of MsPimA complexed with GDP-Man (Protein Data Bank code 2GE) (17)) as a template. Sequence alignment was carried out manually to match functionally conserved residues, predicted secondary structures, and hydrophobicity profiles. Secondary structures were predicted using the Jpred program (18). The models were assessed by the VERIFY 3D program.

RESULTS AND DISCUSSION

MsPimB’ Catalyzes In Vitro the Transfer of a Manp Residue to the 6-Position of the Myo-Ins Ring of PI—With the goal of determining the function of the mycobacterial PimB’ enzyme, a recombinant form of the M. smegmatis protein (MsPimB’) with a C-terminal histidine tag was produced in E. coli BL21(DE3)pLysS and purified to near homogeneity (supplemental Fig. 1S). As had been the case with the M. tuberculosis PimA protein earlier (5, 14, 17), attempts to produce the PimB’ enzyme from M. tuberculosis yielded relatively small amounts of soluble protein compared with the M. smegmatis version, and these efforts were thus not pursued further.

ManT assays were then run using different combinations of the purified MsPimA and MsPimB’ enzymes. When commercial liver PI and GDP-[14C]Man served as the acceptor and donor substrates in the assay, purified MsPimA (14) catalyzed the formation of PIM1 (mannolipid 1, Fig. 1). Unexpectedly, the formation of a 14C-labeled mannolipid with an Rf similar to that of PIM1 was also observed when purified MsPimB’ was used as the source of enzyme in the assay (mannolipid 2, Fig. 1). To further characterize mannolipids 1 and 2, nonradioactive products were purified by preparative TLC from reaction mixtures in which cold GDP-Man replaced GDP-[14C]Man. MALDITOF-MS analyses in the negative ion mode confirmed compounds 1 ([M – H]−, m/z = 1047.60) and 2 ([M – H]−, m/z = 1047.65) as PIM1 molecules (the [M – H]− m/z value of the commercial liver PI is 885.53) (supplemental Fig. 2S). MsPimB’ thus has the ability in vitro to transfer Manp from GDP-Man onto PI, generating PIM1.

A combination of one-dimensional and two-dimensional NMR was then used to determine the position at which the Manp residues were attached to myo-Ins in mannolipids 1 and 2 (for details see supplemental material and supplemental Figs. 3S–6S) (19–21). As depicted in Fig. 2, the 1H NMR spectra of mannolipid 1 shows one peak at 5.14 ppm assigned to the α-anomeric proton of the Manp residue attached to position 2 of myo-Ins. The 1H and 13C chemical shift values of mannolipid 3 is exactly comparable with that of mannolipid 1, and therefore compound 3 was also assigned to 2-linked PIM1. In the spectra of compound 2, the peak at 5.072 ppm was assigned as the α-anomeric proton of the Manp residue attached to position 6 of myo-Ins. The 1H NMR spectra of mannolipid 4 shows two distinct peaks at 5.129 and 5.046 ppm assigned to α-anomeric protons of two Manp residues attached to the 2- and 6-positions of myo-Ins.

For the first time, direct evidence arising from the use of purified enzymes was thus provided that MsPimA catalyzes the transfer of a Manp residue from GDP-Man to the 2-position of the myo-Ins ring of PI, and MsPimB’ catalyzes the transfer of a Manp residue to the 6-position.
Sequential Order of the Mannosylation Reactions Leading to the Formation of PIM2 from PI and GDP-Man—The simultaneous addition of purified MsPimA and MsPimB’ (1:1, w/w) to the reaction mixture described above yielded two products, mananolipid 3 and mananolipid 4 (Fig. 1). MALDI-TOF-MS analyses in the negative ion mode confirmed compound 3 ([M - H]−, m/z = 1047.60) as PIM1 and compound 4 as PIM2 ([M - H]−, m/z = 1209.71) (supplemental Fig. 2S). From this experiment, it can thus be concluded that MsPimA and MsPimB’ are sufficient for the formation of PIM2 from PI and GDP-Man to occur. The fact that no PIM3 or more mannosylated products were formed in the reaction even after prolonged incubation times further indicated that MsPimA and MsPimB’ are unable to mannosylate PIM products beyond PIM2. Thus MsPimA and MsPimB’ appear to each catalyze the transfer of one single Manp residue.

To determine the sequence of the reactions leading to the formation of PIM2, two independent assays were carried out in which purified MsPimA and MsPimB’ were added sequentially to the reaction mixture. In one of the assays, MsPimA was added first to a reaction mixture containing PI and GDP-Man.[C14]Man. After 2 h of incubation, one-half of the reaction was stopped by the addition of CHCl3/CH3OH (2:1), and the other half was incubated at 60 °C for 15 min to inactivate the enzyme. Purified MsPimB’ was then added to the heat-inactivated assay mixture, and the reaction allowed to proceed overnight at 37 °C. In the second assay, MsPimB’ was added first to the reaction mixture, then inactivated as described above, and MsPimA finally added. That both MsPimA and MsPimB’ were inactivated by heat treatment was verified by running independent assays with each of the purified enzymes (supplemental Fig. 7S). Consistent with our previous results, both MsPimA and MsPimB’ catalyzed the transfer of a Manp residue from GDP-Man to PI to form PIM1 (Fig. 3A). The subsequent addition of MsPimB’ to the MsPimA reaction mixture clearly led to the synthesis of [14]C-labeled PIM2 (Fig. 3A). In striking contrast, the addition of MsPimA to the MsPimB’ reaction mixture only resulted in the stimulation of PIM1 production with no detectable formation of PIM2 (Fig. 3A). We thus conclude from this experiment that although MsPimB’ recognizes the PIM1 product of MsPimA (with an α-1,2-linked Manp residue on the myo-Ins ring; Fig. 2) as an acceptor substrate, MsPimA is unable to transfer a Manp residue onto a PIM1 product bearing an α-1,6-linked Manp residue.

With the transfer of Manp from GDP-Man onto PI catalyzed by MsPimB’ occurring 53 times slower than both the MsPimA-dependent transfer of Manp onto PI and the MsPimB’-dependent addition of Manp onto PIM1 (Fig. 3, B and C), it is clear that the different activities of the two enzymes with PI and, subsequently, PIM1 acceptors dictate the order in which the mannosylation of PI and PIM is to occur under physiological conditions. In further support of this assumption, the PIM1 product
mannolipid 3, Fig. 1) formed in a competition assay, where equal amounts of MsPimA and MsPimB \( /H11032 \) were used as enzyme sources, exclusively consisted of \( /H9251 \)-1,2-linked Manp residues, as opposed to the expected mixture of \( /H9251 \)-1,2- and \( /H9251 \)-1,6-linked Manp residues if both enzymes had transferred Manp onto PI with comparable efficiencies (Fig. 2).

MsPimB Stimulates the Production of Ac1PIM2 in M. smegmatis Membrane Preparations—When membranes prepared from \( M. \) smegmatis mc\( ^{2155} \) were used as a source of phospho-(glyco)lipid acceptor, the addition of purified MsPimA clearly stimulated the synthesis of PIM1, accompanied by the accumulation of small amounts of Ac1PIM1 (Fig. 4, lane 2). The addition of MsPimB to the membrane preparations, in contrast, led to an even greater accumulation of a compound (mannolipid 5) with \( Rf \) properties similar to that of Ac1PIM2 (Fig. 4, lane 4). MALDI-TOF MS and NMR analyses confirmed the identity of this product as Ac1PIM2 containing two C16 and one C19 fatty acyl chains (\([/M-\text{H}]^-; m/z = 1413.88\) (supplemental Fig. 2S) (21), among which two fatty acyl chains are carried by the glyceroi moiety and one acyl chain is attached to Manp residue located at position 2 of the myo-Ins ring (supplemental Fig. 8S). The acylation of the Manp residue transferred by PimA is thought to result from the action of the acyltransferase encoded by MSMEG_2934 in \( M. \) smegmatis mc\( ^{2155} \) (13).

Overall, the abundant de novo synthesis of Ac1PIM2 in the assay mixture containing purified MsPimB’ (Fig. 4, lane 4) suggests that significant amounts of PIM1 are available in the membranes of \( M. \) smegmatis or that the synthesis of this acceptor substrate is stimulated by the addition of purified MsPimB’ to the reaction mixture. This observation and the fact that radio-labeled Ac1PIM2 was on the contrary not detectable in the assay mixture in which only purified MsPimA was added (Fig. 4, lane 2) suggest that the physiological amounts of MsPimB’ present in the membranes of \( M. \) smegmatis may be rate-limiting in the formation of PIM\(_2\)/Ac1PIM\(_2\). On the other hand, with almost
FIGURE 5. Proposed pathway for the early steps of PIM biosynthesis in mycobacteria. The two pathways originally proposed for the biosynthesis of Ac₃PIM₂ in mycobacteria are shown. (i) PI is mannosylated to form PIM₁. PIM₁ is then mannosylated to PIM₂, which is acylated to form Ac₃PIM₂. (ii) PIM₁ is first acylated to Ac₁PIM₁ and then mannosylated to Ac₁PIM₂. Our experimental evidence indicates that although both pathways might co-exist in mycobacteria (13), the sequence of events PI → PIM₁ → PIM₂ → Ac₃PIM₂ is favored. As an important part of the literature concerning PIM studies refers to the nomenclature based on the M. tuberculosis H37Rv sequences, the Rv numbers of the proteins are also included.
all of the PIM₁ product of MsPimB⁺ being instantly converted to Ac₁PIM₂ (Fig. 4, lane 4), the activity of the acyltransferase does not seem to be limiting in the membranes of M. smegmatis. In fact, saturation of this enzyme only became clearly visible when both purified MsPimA and MsPimB⁺ were added to the reaction mixture, resulting in the accumulation of abundant quantities of PIM₁ and PIM₂ (Fig. 4, lane 3). Finally, the quasi-exclusive occurrence of PIM₂'s under their acylated form (Ac₁PIM₂) in the assay where MsPimB⁺ was added (Fig. 4, lane 4), whereas the product of the reaction catalyzed by MsPimA essentially occurred as PIM₁ (i.e. with no acylation on the Manp residue) (Fig. 4, lane 2), strongly suggests that the acyltransferase MSMEG_2934 preferentially acylates PIM₂ over PIM₁. Thus, despite MSMEG_2934 displaying acyltransferase activity on both PIM₁ and PIM₂ in vitro (13), it is likely that under physiological conditions the preferred pathway to Ac₁PIM₂ involves the transfer of both mannosyl residues onto PI prior to the acylation of the α-1,2-linked Manp residue.

Revised Model for the Early Steps of PIM Biosynthesis—Based on present experimental evidence, a revised model for the early steps of PIM biosynthesis is presented in Fig. 5. As inferred from previous studies (5, 22–24) and now unambiguously demonstrated, MsPimA is the first enzyme engaged in the pathway. It is responsible for transferring a Manp residue from GDP-Man onto the 2-position of the myo-Ins ring of PI to form PIM₁. MsPimB⁺ then transfers a second Manp residue from the same sugar donor to the 6-position of the myo-Ins ring of PIM₁, yielding PIM₂. Finally, the acyltransferase MSMEG_2934 acylates the Manp residue transferred by PimA to yield one of the major forms of PIM species found in mycobacteria, Ac₁PIM₂.

**MsPimB⁺ Is Essential for the Growth of M. smegmatis**—To investigate the essentiality or, on the contrary, possible redundancy of the ManT PimB⁺ in mycobacteria, a MsPimB⁺ (MSMEG_4253) conditional mutant of M. smegmatis mc²155 was constructed. The methodology employed relies upon a suicide plasmid harboring the counter-selectable marker sacB to achieve allelic replacement, and a replicative temperature-sensitive plasmid (pCG76) to express a rescue copy of the gene of interest. Briefly, clones having undergone single crossover at the Mspsmb locus were first selected upon plating of mc²155/pJQMspsmb/KX transformants on LB-Kan plates at 37 °C. Single crossover recombinants were grown in LB-Kan broth and then plated onto sucrose containing plates at 30 or 37 °C to select for allelic exchange mutants. No knock-out mutants were isolated at this stage strongly suggesting that Mspsmb⁺ was essential for growth regardless of the temperature used. To confirm this assumption, a conditional mutant of M. smegmatis was constructed. A temperature-sensitive rescue plasmid carrying a wild type copy of the Mspsmb⁺ gene, pCGMspsmb⁺, was introduced in one of the single crossover recombinants, and the resulting merodiploids were plated onto LB-Kan-sucrose plates at 30 °C. Candidate conditional mutants were obtained in which allelic replacement at the chromosomal Mspsmb⁺ locus was confirmed by PCR (Fig. 6A). The conditional mutants grew normally at 30 °C in liquid broth or on plates, a temperature at which pCGMspsmb⁺ replicates, but lost viability at 42 °C where the rescue plasmid is lost (Fig. 6B). Results thus indicated that Mspsmb⁺ is essential for the growth of M. smegmatis under the experimental conditions used. Therefore, despite the interchangeability of the M. tuberculosis PimB and PimB⁺ enzymes expressed in C. glutamicum in cell-free assays (12), the function of MsPimB⁺ cannot be compensated by any other ManTs, including MsPimB (MSMEG_1113; 75% identical to PimB from M. tuberculosis on a 375-residue overlap) in whole M. smegmatis cells.

**Structural Comparison of MsPimA and MsPimB⁺**—The α-ManT's MsPimA and MsPimB⁺ belong to the large GT4 fam-
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Figure 7. Structural similarity between MsPimA and MsPimB. A, an experimental three-dimensional model of the crystal structure of MsPimA. B, three-dimensional homology model of MsPimA (Protein Data Bank code 2GEJ, see Ref. 17). C, structural alignment of MsPimA and MsPimB. Secondary structure elements of the MoPimA three-dimensional structure are shown above the protein sequence. Wavy lines indicate disordered regions in the three-dimensional structure. The basic cluster in helix a2, which is proposed to be involved in membrane interaction, is highlighted in blue. Identical residues are shown in an orange background, and homologous residues are shown in a yellow background. Residues involved in the binding of GDP-Man and PI are denoted with solid and open circles, respectively.

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FIGURE 7. Structural similarity between MsPimA and MsPimB. A, an experimental three-dimensional model of the crystal structure of MsPimA. B, three-dimensional homology model of MsPimA (Protein Data Bank code 2GEJ, see Ref. 17). C, structural alignment of MsPimA and MsPimB. Secondary structure elements of the MoPimA three-dimensional structure are shown above the protein sequence. Wavy lines indicate disordered regions in the three-dimensional structure. The basic cluster in helix a2, which is proposed to be involved in membrane interaction, is highlighted in blue. Identical residues are shown in an orange background, and homologous residues are shown in a yellow background. Residues involved in the binding of GDP-Man and PI are denoted with solid and open circles, respectively.
emphasizes their interest as novel targets for anti-tuberculosis chemotherapeutic agents.

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