PimF, a Mannosyltransferase of Mycobacteria, Is Involved in the Biosynthesis of Phosphatidylinositol Mannosides and Lipoarabinomannan

David C. Alexander, Joses R. W. Jones, Tracy Tan, Jeffrey M. Chen, and Jun Liu

From the Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Phosphatidylinositol mannosides (PIMs) and their related molecules lipomannan (LM) and lipoarabinomannan (LAM) are important components of the mycobacterial cell wall. These molecules mediate host-pathogen interactions and exhibit immunomodulatory activities. The biosynthesis of these lipoglycans is not fully understood. In this study, we have identified a mycobacterial gene (pimF) that is involved in the synthesis of PIMs. We have named this gene pimF. Transposon mutagenesis of pimF of Mycobacterium marinum resulted in multiple phenotypes, including altered colony morphology, disappearance of tetracyl-PIM₇, and accumulation of tetraacyl-PIM₅. The synthesis of LAM and LM were also affected. In addition, the pimF mutant exhibited a defect during infection of cultured macrophage cells. Although the mutant was able to replicate and persist within macrophages, the initial cell entry step was inefficient. Transformation of the M. marinum mutant with the pimF homolog of Mycobacterium tuberculosis complemented all of the above mentioned phenotypes. These results provide evidence that PimF is a mannosyltransferase. However, sequence analysis indicates that PimF is distinct from mannosyltransferases involved in the early steps of PIM synthesis. PimF catalyzes the formation of high molecular weight PIMs, which are precursors for the synthesis of LAM and LM. As such, this work marks the first analysis of a mannosyltransferase involved in the later stages of PIM synthesis.

The mycobacterial cell wall modulates interactions between the tubercle bacillus and its environment. The cell wall consists of a covalently bound structure, the mycolic acid-arabinogalactan-peptidoglycan complex, and a variety of free lipids that complement the mycolate residues to form an asymmetric bilayer (1–3). Among the cell wall-associated lipids, phosphatidylinositol mannosides (PIMs) and their multiglycosylated counterparts lipomannan (LM) and lipoarabinomannan (LAM), have emerged as major factors in mediating host-pathogen interactions and are presumed to be necessary for the survival and persistence of the pathogen within the host (4). LAM has been implicated in various immunomodulatory effects, including the down-regulation of cell-mediated immunity (5). In addition, the manno-capped LAM found in the slowly growing mycobacteria, including Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis, and Mycobacterium avium, has been shown to bind cell surface receptors of macrophages and dendritic cells and aid invasion of host cells (6–8). PIMs are known to mediate the attachment of M. tuberculosis to nonphagocytic cells and have been implicated in the recruitment of natural killer T cells, which affects the granulomatous response (9, 10).

Mycobacterial LAMs are lipoglycans composed of three structural components: the membrane anchor, which is a mannosyl-phosphatidyl-myo-inositol; the backbone, which consists of two homopolysaccharides, mannopyranose (Manp) and arabinofuranose (Araf); and the capping motif, which varies among mycobacterial species (11, 12). At least three types of capping motifs have been described, including manno-capped LAM found in slowly growing mycobacteria, PILAM found in the fast growing mycobacteria Mycobacterium smegmatis and Mycobacterium fortuitum (13), and AraLAM found in Mycobacterium chelonae (14).

Despite their important biological functions, little is known about the biosynthesis of PIMs, LM, and LAM. Structural studies indicate that PIMs, LM, and LAM all share a conserved mannosyl-phosphatidyl-myo-inositol anchor, suggesting that they are metabolically related (12). Early studies with PIMs led to the proposed sequence of PI → PIMs → LM → LAM, which is supported by a growing body of biochemical and genetic evidence (11, 12). PIMs are predominantly found as di- and hexamannoside forms, PIM₂ and PIM₄, respectively (10, 15). The formation of PIM₂ is catalyzed by two distinct mannosyltransferases, PimA and PimB (16, 17). Using GDP-mannose as the sugar donor, PimA catalyzes the addition of one Manp to the 2-position of the myo-inositol of PI to form PIM₁. PimB catalyzes the addition of another Manp to the 6-position to yield PIM₂. Further mannosylation is mediated by PimC (18), which transfers Manp from GDP-Man to PIM₂, yielding PIM₃. It has been proposed that PIM₂ is further mannosylated to form high molecular weight PIMs, which leads to the formation of LM. LM is then glycosylated with arabinan to form LAM (12). However, genes involved in these subsequent glycosylation steps have not been identified, and details of this pathway remain highly speculative.

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LAM and LM are characterized by a linear α(1→6)-linked Man backbone punctuated by α(1→2) Man side chains. The PIM intermediates between PIM₂ and LM/LAM are thought to be PIM₂ → PIM₃, so-called higher PIMs, since they all lack the α(1→2) Man side chains (19, 20). PIM₃, the other abundant class of PIMs, appears to be a terminal product, because it contains linear α(1→2)-linked Man, which is not found in LM and LAM (20).

In this study, we have identified a mannosyltransferase that catalyzes the synthesis of higher PIMs and is involved in the biosynthesis of LAM and LM. We have demonstrated, for the first time, that disruption of PIMs affects LAM and LM synthesis, which provides direct evidence for the biosynthetic relationship of PIMs, LM, and LAM. In accordance with the nomenclature that has been used in the literature, we have called this enzyme PimF.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Mycobacterium marinum strain 1218R (ATCC 927) was obtained from L. P. Barker (NIAID, National Institutes of Health). Strain MRS2521 (pimF::emyCmar) was generated by transposon mutagenesis of 1218R. M. marinum cells were grown at 30 °C in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol and 10% oleic acid-albumin-dextrose complex (Difco) or on Middlebrook 7H11 agar (Difco) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose complex. Escherichia coli strain DH5α was used for routine manipulation and propagation of plasmid DNA. E. coli strain DH5α λ pir116 (21) was used for isolation of transposon-containing plasmids. Antibiotics were added as required: kanamycin, 50 μg/ml for E. coli; and 25 μg/ml for M. marinum; hygromycin B, 150 μg/ml for E. coli; and 75 μg/ml for M. marinum.

Generation and Screening of M. marinum ΔemyCmar Insertion Library—Propagation of the ΔemyCmar transposon phage and preparation of phage lysates have been described previously (22). For phage infection, M. marinum 1218R cells were washed and resuspended in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgSO₄, 2 mM CaCl₂. Phage were added at a multiplicity of infection of 10:1 and incubated at 37 °C for 3 h to allow infection to occur. Bacteria were then plated on Middlebrook 7H11 agar supplemented with kanamycin and incubated at 30 °C. Kanamycin-resistant (i.e. transposon-containing) M. marinum colonies were patched onto Middlebrook 7H11 agar to obtain a library of 7680 (i.e. 80 plates × 96 colonies/plate) colonies. Colonies with unusual morphology were identified by visual inspection.

Localization of the ΔemyCmar Insertion—M. marinum MRS2521 chromosomal DNA was isolated by standard methods. Total chromosomal DNA was cleaved with BamHI, a restriction endonuclease that does not cut within the Mycobacterium element. Such digestion generated a restriction fragment containing the kanamycin resistance cassette and flanking chromosomal DNA. Self-ligation of this restriction fragment generates a plasmid that can replicate in E. coli strains containing the pir gene. Digested DNA was self-ligated with T4 DNA ligase and transformed into competent E. coli DH5α λ pir116. Plasmid DNA was isolated from E. coli transformants, Oligonucleotide primers MAR1 (5′-CCCCGAAATTGCCA-CGTGAAAAGGCC-3′) and MAR2 (5′-CGTCTCTCTGCTTATTGATCG-TATCG-3′) were used to determine the DNA sequence of the Mycobacterium/ chromosome junction. These DNA sequences were compared with the genome sequences of M. marinum and M. tuberculosis at the Sanger Institute (www.sanger.ac.uk) and analyzed with NTI Suite software (Informax).

Molecular Cloning—The pPMT plasmids were generated by cloning DNA fragments from the M. tuberculosis H37Rv BAC library (obtained from S. Cole, Institut Pasteur, Paris) (23) into the E. coli-Mycobacterium shuttle vector pNDV1, which contains a hygromycin resistance cassette and the β-lactamase gene. Plasmid pNDV1 contains 2.2-kb PstI, 2.9-kb PmaCI, and 5-kb HindIII fragments of BAC58, respectively (see Fig. 2C).

TLC Analysis of Cell Wall Lipids—The apolar and polar lipids were extracted from M. marinum cells (50 mg dry biomass) according to the published procedures (25). These lipids were analyzed by two-dimensional TLC on thin-layer 60 plates (Whatman). Apolar lipids were developed with petroleum ether/ethyl acetate (98:2, three times) in the first dimension and petroleum ether/acetone (98:2) in the second dimension. Polar lipids were separated with chloroform/methanol/water (60:30:6) in the first dimension and chloroform/acetid acid/methanol/water (40:25:3:6) in the second dimension. Lipids were detected by charring with α-naphthol or 5% phosphomolybic acid.

Mass Spectrometry (MS) Analysis—Preparative TLC was performed to isolate lipids of interest for analysis by mass spectrometry. Briefly, polar lipids were separated on a set of four TLC plates that were run concurrently. One plate was stained with α-naphthol spray to determine the locations of lipids. The corresponding areas were scraped from the uncharred plates, dissolved in CHCl₃/Methanol (2:1), and subjected to MS analysis at the Molecular Medicine Research Center, University of Toronto.

MALDI MS spectra were acquired in DE-reflectron mode at positive ion mode on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a 337-nm laser. Acceleration voltage was set at 20 kV, grid voltage at 72%, guide wire at 0.001%, and delay time at 190 ns. MALDI-FSD spectra were acquired at DE-reflectron mode. The time ion selector was preset to the [M + H]+ mass of the analyte. The spectrum was acquired in 10 segments with mirror ratios from 1.0 to 0.06 and then assembled by the instrument software.

Analysis of LAM and LM—To prepare crude extracts of LAM and LM, the delipidated cells (i.e. after the removal of polar and apolar lipids) were extracted three times with 50% ethanol. The ethanol extracts were evaporated to dryness and resuspended in 10 mM Tris-HCl, pH 5.7, 0.2 mM NaCl, 0.5 mM MgSO₄, and 0.25% sodium deoxycholate. Samples were then run on 10% Tricine-SDS-polyacrylamide gels. For visualization of both LAM and LM, gels were silver-stained according to published protocols (25), except the reducing agent dithiothreitol was omitted. Western blotting was also performed to detect LAM, using the monoclonal α-LAM antibody CS-35, obtained from J. Belisle (Colorado State University). This antibody is specific for an arabinofuranosyl-containing epitope found in mycobacterial LAM but does not recognize LM (26).

Infection of J774 Macrophages by M. marinum—The infection assay was performed according to a procedure described previously (27). The mouse macrophage-like cell line J774A.1 (ATCC TIB67) was maintained at 37 °C in 5% CO₂ in RPMI 1640 media with l-glutamine (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen). Prior to infection, J774 cells were seeded into 24-well tissue culture plates (Corning) at a density of 2 × 10⁴ cells/well in 1 ml of RPMI 1640 medium supplemented with 10% FBS and maintained at 32 °C in 5% CO₂. After overnight growth, the J774 cells were infected. Spent medium was replaced with 1 ml of fresh RPMI 1640 medium containing 10% FBS and sufficient mycobacteria to achieve a multiplicity of infection (MOI) of 1 (i.e. one bacterium for one macrophage). The infection medium was replaced with FBS-containing culture medium. After 48 h at 32 °C in 5% CO₂, after which time extracellular bacteria were removed by washing the J774 cells twice with fresh RPMI 1640 containing 10% FBS. Subsequently, cells were incubated at 32 °C in 5% CO₂ in 1 ml of fresh RPMI 1640 media with 10% FBS. The media for these cells were replaced every other day with fresh RPMI 1640 with 10% FBS. On days 0 (3 h), 2, 6, 8, and 10, the infected macrophage monolayers (three wells/strain) were washed twice with fresh RPMI 1640 medium with 10% FBS and then lysed with 0.1% Triton X-100 (Sigma) to release intracellular contents. After 15 min, 1× PBS was added to the lysed cells to give a final volume of 1 ml. The number of intracellular mycobacteria was enumerated by plating appropriate dilutions (1:100, 1:1000, and 1:10,000) on Middlebrook 7H11 agar plates containing appropriate antibiotics. Agar plates were incubated at 32 °C for 7 days, at which time colony-forming units were counted.

RESULTS

Isolation of M. marinum MRS2521 Mutant—One goal of our research is to elucidate the pathways required for biosynthesis of the mycobacterial cell wall. To achieve this, we have taken a simple approach, based on the assumption that defects in certain cell wall components will alter the surface properties of the bacterial cell and give rise to colonies with unusual morphology. To this end, a library of ~8000 M. marinum transposon insertion mutants was generated and visually inspected to identify clones with altered colony morphology. More than 20 dozen mutants have been isolated and partially characterized. Consistent with our goal, we have obtained mutants that exhibit altered mycolic acid composition and show defects in

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peptidoglycan and polyketide synthesis. We recognize that colony morphology is a complex phenotype, and it is difficult to fully understand the structural basis. Nevertheless, our preliminary data and the evidence presented in this paper indicate that this is an effective approach toward the understanding of cell wall biosynthesis.

The mutant described here, MRS2521, produced a distinctive “corona,” a wide, filamentous, translucent border around the colony (Fig. 1). MRS2521 exhibited normal growth rate on solid media or liquid culture, and cells appeared normal when examined by light microscopy (data not shown). The sensitivity of the mutant to antibiotics, including rifampicin, streptomycin, isoniazid, and ethambutol, was the same as that of the wild type cell (data not shown).

Identification of pimF Gene—To identify the gene that had been disrupted by the transposon in MRS2521, we performed molecular characterizations of the mutant as described under “Experimental Procedures.” DNA sequencing and analysis of the MycoMar/M. marinum chromosomal junction revealed that the transposon had inserted at a TA dinucleotide, 412 bp downstream from the ATG start codon of a previously uncharacterized open reading frame, which we now call pimF (Fig. 2A). Sequence analysis revealed that the predicted protein product is homologous (78% identity, 86% similarity in amino acid sequence) to the Rv1500 of M. tuberculosis H37Rv (Fig. 2D). Inspection suggested that the pimF region of M. marinum was analogous to the Rv1497 → Rv1505 region of the M. tuberculosis genome (Fig. 2E). In addition to Rv1500, homologs to Rv1497, Rv1501, Rv1502, Rv1503, Rv1504, and Rv1505 are present. In all organisms, pimF/Rv1500 and Rv1501 appear to be co-transcribed. However, the M. marinum region is ∼11 kb larger than the corresponding portion of the M. tuberculosis genome and includes (at least) nine open reading frames not found in M. tuberculosis (Fig. 2A and B). Other M. tuberculosis strains, including clinical isolates CDC1551 and 210, and M. bovis strain AF2122/97 contain a pimF homolog that is identical to Rv1500. Interestingly, a pimF homolog was not found in the complete or nearly complete genome data bases of M. leprae, M. avium, M. paratuberculosis, or M. smegmatis.

Analysis of the Mutant Cell Wall Lipids—Sequence analysis reveals that PimF belongs to a large family of glycosyltransferases, designated Family 2 in the CAZY classification (ambl-cns-mre.fr/CAZY) and Group 2 in the Pfam data base. These diverse glycosyltransferases transfer sugar from various donors, including GDP-mannose, UDP-glucose, UDP-N-acetyl-galactosamine or CDP-abequose, to a range of substrates, including cellulose, dolichol phosphate, and teichoic acids. We postulated that PimF might be involved in the biosynthesis of a specific cell wall component, since the disruption of pimF affects colony morphology. The cell wall of M. marinum contains various glycolipids that can be differentially extracted by organic solvents, including phenolic glycolipids, phthiocerol dimycocerosate, triacylglycerol, and PIMs (1). To determine if the synthesis of these lipids was affected in the mutant, we applied established two-dimensional TLC developing systems (25) to examine these lipids. In all experiments, lipids extracted from WT cells under the same conditions were analyzed in parallel. Analysis of apolar lipids, including phenolic glycolipids, triacylglycerol, and phthiocerol dimycocerosate, did not reveal any difference between the mutant and the WT strains (data not shown). However, a distinct profile of polar lipids was observed for the mutant (Fig. 3). The mutant accumulated large amounts of one lipid (Fig. 3, A and B, spot 1, arrowhead) and failed to produce another (Fig. 3, A and B, spot 2, arrow), suggesting a precursor-product relationship between these two lipids. Previous two-dimensional TLC analyses of polar lipids of M. tuberculosis and M. bovis BCG followed by structural determinations by MS and NMR established that these lipids represented various PIMs that differ in the degree of mannosylation and acylation (10, 15, 18). To confirm these structural predictions, spots 1 and 2 were purified by preparative TLC and subjected to MS analysis. Fig. 4A shows the MALDI-TOF spectra of spot 1, which consists of a group of molecular ions that differ in size by increments of 14, the molecular mass of CH2=, suggesting that these ions belong to a homologous series differing only in the acyl chain length. The molecular ion at m/z 2308 was further analyzed by MALDI-PSD, and the resulting fragment ions are shown (Fig. 4B). Based on these data, we propose that spot 1 represents tetraacyl-PIM5 (Fig. 5). Similarly, spot 2 was analyzed by MALDI-TOF and MALDI-PSD (Fig. 4, C and D). The observation that the ion of spot 2 at m/z 2632 is two mannoses larger than m/z 2308 of spot 1 (2308 + 2 × 162 = 2632) and that the MALDI-PSD spectrum of m/z 2632 is nearly identical to that of m/z 2308 (Fig. 4, compare B and D) suggest that spot 2 represents tetraacyl-PIM7 (Fig. 5).

Three other lipids on the two-dimensional TLC, which were not affected in the mutant, were also analyzed by MS (data not shown) and identified as Ac5PIM2, Ac5PIM6, and Ac4PIM6 (Fig. 3A), which are consistent with previous studies (10, 18).

Complementation of the MRS2521 Mutant—To confirm that interruption of pimF was responsible for the altered lipid profile and unusual colony morphology of MRS2521, the mutant was transformed with plasmids containing the homologous M. tuberculosis Rv1500 gene. Plasmids pPMT1, pPMT2, and

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pPMT3, which contain Rv1500, Rv1500 plus Rv1501, and Rv1498 to Rv1502, respectively (Fig. 2C), restored the wild type colony morphology (Fig. 1C). These plasmids also complemented the two-dimensional TLC lipid profile of the mutant (i.e. the Ac4PIM5 accumulated in the mutant was converted to Ac4PIM7) (Fig. 3, D–F). In mutants transformed with pPMT1, slightly more AC4PIM5 remained. It is not clear if this is due to differences in PimF expression level in these constructs. However, mutant transformed with the cloning vector alone failed to restore the WT colonial morphology (not shown) or the lipid profile (Fig. 3C). Taken together, these results suggest that PimF is a mannosyltransferase that mediates the biosynthesis of PIMs, specifically the synthesis of PIM7 from PIM5.

Analysis of LAM and LM Synthesis—As mentioned above, PIMs are thought to be precursors of LM and LAM on the basis of the structural resemblance of these molecules (12). However, direct evidence to demonstrate this metabolic relationship has yet to be obtained. Disruptions of genes encoding mannosyltransferases involved in the early steps of PIM synthesis had failed to block the synthesis of LAM and LM (16, 18), and consequently, LAM- or LM-defective mutants have not been isolated. Such mutants are highly desirable, since they would allow the direct evaluation on the role of these lipoglycans in pathogenesis and virulence of mycobacteria.

So far, PimF is the only mannosyltransferase identified involving in the later steps of PIM synthesis (i.e. the synthesis of higher PIMs). Since these PIMs are more immediate precursors of LAM and LM, it is likely that disruption of pimF could affect the synthesis of LAM and LM. To test this, we examined the production of LAM and LM in the mutant and compared it with that of the WT in parallel experiments. Both LAM and LM can be readily detected by gel electrophoresis, and the result is shown in Fig. 6. In the mutant strain, both LAM and LM were detected upon visualization by silver staining (Fig. 6A). Yet, in

Fig. 2. Genetic organization of pimF/Rv1500 regions. M. marinum pimF region (A) and the corresponding M. tuberculosis H37Rv Rv1500 region (B). The vertical arrow indicates the site of transposon insertion. The block arrows represent open reading frames: homologous in M. marinum and M. tuberculosis (black); similar in M. tuberculosis (striped); unique to M. marinum (white); and unique to M. tuberculosis (gray). DNA restriction fragments from M. tuberculosis present in pPMT plasmids are indicated with thick bars (C). H, HindIII; P, PstI; M, PstClI. D, sequence alignment of PimF of M. marinum and Rv1500 of M. tuberculosis.
comparison with the WT strain, LAM and LM levels appeared to be somewhat decreased (Fig. 6A). Western blot using antibody that recognizes LAM also showed that the amount of LAM was decreased in the mutant (Fig. 6B). Furthermore, levels of LAM and LM were restored in mutants transformed with the pimF homolog of M. tuberculosis (Fig. 6, A and B). These results suggest that PimF is involved in the synthesis of LAM and LM.

Infection of Murine Macrophages with the pimF Mutant—Pathogenic mycobacteria including M. tuberculosis selectively invade the alveolar macrophage, one of the first cells encountered by the bacteria after their inhalation. Once inside of macrophages, the bacilli are capable of modifying the phagosomal compartment in order to enhance their own intracellular survival, and consequently, the mycobacterial phagosome is arrested at an early stage of maturation and only mildly acidified (29–31). It has been recently suggested that the LAM of M. tuberculosis causes phagosome maturation arrest by inhibiting a cascade consisting of cytosolic Ca\(^{2+}\) transients, calmodulin, phosphatidylinositol 3-kinase hVPS34, and EEA1 and that LAM could act as a trafficking toxin that contributes to the survival and persistence of the bacilli in host macrophages (32–34).

Given the phenotype of the pimF mutant, it is logical to determine whether the intracellular survival of the mutant is affected. Macrophage J774 cells were infected with the wild-type parental strain 1218R, the MRS2521 mutant, and the mutant complement MRS2521/pPMT2, at an MOI of one bacterium for one macrophage for 3 h at 32 °C. Hereafter, the infections were monitored by counting viable intracellular bacteria over a 10-day period. All experiments were performed in parallel and the result is shown in Fig. 7. It appears that all three strains exhibited comparable rates of replication in macrophages during the course of infection (Fig. 7A). However, although equal numbers of bacterial cells were used to infect macrophages, at day 0 (3 h postinfection), the number of pimF mutants recovered from macrophage was only 50% of that of the WT or mutant complement (Fig. 7B). This difference was statistically significant according to the Student’s t test (p < 0.05). When the infection was performed at an MOI of 10 (i.e. 10 bacterium for one macrophage), a similar result was obtained (Fig. 7C), and the difference between the mutant and the WT or the mutant complement was also statistically significant (Student’s t test, p < 0.05). These results indicate that the pimF mutant was less efficient entering macrophages. This is in agreement with the lower amounts of LAM and LM present in the mutant, resulting in a decreased uptake by phagocytosis. The ability of the mutant to replicate and persist inside of macrophages, however, does not appear to be compromised.

**DISCUSSION**

In this study, we have identified a mycobacterial gene, Rv1500/pimF, encoding a mannosyltransferase involved in the synthesis of PIMs. PimF is the first enzyme involved in a later
step of PIM synthesis that has been described so far. Specifically, PimF mediates the transfer of mannose to Ac₄PIM₅, which leads to the formation of Ac₄PIM₇. Several lines of evidence support this conclusion. First, disruption of pimF by transposon mutagenesis blocked the synthesis of Ac₄PIM₇ and resulted in the accumulation of its precursor, Ac₄PIM₅. Trans-
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formation of the mutant with plasmids containing pimF restored the production of Ac4PIM7. Second, sequence analysis indicates that PimF is distinct from the three mannosyltransferases involved in PIM synthesis that have been identified so far, PimA, -B, and -C (16–18). These mannosyltransferases belong to Family 4 of the CAZy classification of glycosyltransferases and Group 1 of the Pfam data base. They contain the EX-E motif that is present in a number of bacterial glycosyltransferases including other mannosyltransferases. It was suggested that the EX-E motif is involved in sugar nucleotide binding (35). Biochemical evidence has shown that PimA, -B, and -C all use GDP-Man as the sugar donor and mediate the early steps of PIM synthesis (i.e. PI → PIM1 → PIM2 → PIM3, respectively) (16–18). In contrast, PimF does not contain the EX-E motif (Fig. 2D) and belongs to a different family of glycosyltransferases, as mentioned above. This difference suggests that PimF may utilize a different mechanism to carry out its enzymatic reaction, which could account for its unique substrate specificity (i.e. catalyzing the mannosylation of high molecular weight PIMs). The sugar donor has not been identified, but it is possible that PimF utilizes a different mannose donor than GDP-Man. It has been suggested that polyprenol monophosphomannose is the sugar donor employed for synthesis of high molecular weight PIMs and LAM (36). Although the exact biochemical mechanism of the PimF is unknown, it is conceivable that PimF could mediate the sequential addition of two mannoses to Ac3PIM6 to form Ac4PIM7 or, alternatively, one mannose to form Ac3PIM6, which is then converted to Ac4PIM7 by a different enzyme. Further biochemical studies with purified PimF protein will help to address this question.

Our data also suggest that PimF is involved in the biosynthesis of LM and LAM. The amount of Ac3PIM6 accumulated in the pimF mutant is at least 5 times more than the level of Ac3PIM7 detected in the WT strain, judging from the two-dimensional TLC data (Fig. 3, A and B), suggesting that Ac3PIM7 is not a terminal product but instead an intermediate in the biosynthetic pathway of LAM and LM. Consistently, the levels of LAM and LM in the mutant were decreased, and such phenotype was complemented in the mutant strain transformed with pimF gene (Fig. 6). Taken together, these results indicate that PimF is a mannosyltransferase involved in the biosynthesis of LM and LAM. Fig. 8 shows a proposed model of the biosynthesis of PIMs, LM, and LAM, illustrating the step that PimF could mediate. The observation that inactivation of pimF did not affect the synthesis of PIM6 species (Fig. 3) also supports this conclusion. PIM6 is one of the most abundant PIM species found in mycobacteria. PIM6 is considered to be a terminal product and not a precursor for the synthesis of LM and LAM, since it contains linear α(1→2)-linked Man, which is not present in LM or LAM (20). We detected two forms of PIM6 in M. marinum, Ac3PIM6 and Ac4PIM6, and neither was affected by the disruption of pimF (Fig. 3), which in turn implies that PimF participates in a pathway that has diverged from the one leading to PIM6 synthesis. In agreement with our data, a recent model based on biochemical studies also depicted PIM6 as a branch point at which the pathways of PIM6 and LAM/LM...
Fig. 8. Proposed pathways for the biosynthesis of the PIMs, LM, and LAM. Only the late steps of PIM synthesis are shown. PimA and PimB catalyze the early steps of PIM synthesis (i.e., PI → PIM → PIM₂, respectively) (not shown). PimF is the only enzyme involved in a later step of PIM synthesis identified so far. The heterogeneity of PIMs is illustrated. Acyltransferases (AcylT) could mediate the acylation of the PIMs, resulting in PIMs of various degrees of acylation (e.g., di- to tetraacylated PIM₂ and PIM₃), which then can be mannosylated by a family of mannosyltransferases (ManT).
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synthesis diverge (37). Currently, we do not know the nature of mannan linkage in Ac3PIM or Ac4PIM, but it is likely that they, like LAM and LM, contain linear α(1→6)-linked rather than α(1→2)-linked Man.

Disruption of pimF did not completely block the synthesis of LAM and LM. This is not entirely surprising, since it suggests that there is a redundancy in the step mediated by PimF. Such redundancy has been observed in the early steps of PIM synthesis. Although pimA is essential, pimB and pimC are both dispensable (16, 18). Inactivation of either pimB or pimC did not affect cell growth or PIM synthesis; nor did it affect the synthesis of LM and LAM (18). Moreover, there are a number of glycosyltransferases in the M. tuberculosis genome, including the uncharacterized Rv0539, Rv1208, and Rv3631 that belong to the same family of glycosyltransferases as PimF. In addition, PIMs and LAM are highly heterogeneous molecules, which vary in the degree of glycosylation and acylation (11, 12). The predominant molecules also vary with mycobacterial species. The complete structures of native PIM2 and PIM4 molecules were determined recently, which unambiguously established the existence of mono- to tetraacylated molecules in M. tuberculosis and M. bovis BCG (10, 15). An acyltransferase (Rv2611c) that mediates the acylation of PIM1 and PIM2 was identified recently (38) (Fig. 3). We also detected, at least two species of PIM1 in M. marinum, Ac3PIMΔ, and Ac4PIMΔ (Fig. 3). Consistent with this, an early study showed that LAM and LM of M. tuberculosis could be resolved into species carrying two to four fatty acyl chains on hydrophilic interaction column (39). Thus, it is conceivable that PimF is involved in the synthesis of only a subset of LM and LAM (i.e. one that carries four fatty acyl chains to their PIM moiety). Other members of the same family of glycosyltransferases could catalyze a similar reaction but with substrates that are acylated at different degree (Fig. 8).

The fact that a PimF homolog is absent in M. leprae, M. avium, M. paratuberculosis, and M. smegmatis suggests that these organisms may have PIMs and LAM species that differ in the degree of acylation and demand enzymes with different substrate specificity.

Our data show that the ability of the pimF mutant to enter/ invade macrophages is compromised relative to the wild type, which is in agreement with the decreased amounts of LAM and LM in the mutant. LAM is known to interact with mannose receptor and CD14, two of the receptors employed by mycobacteria for entering host macrophages (6). The entry of the mutant to macrophages was not completely abolished, which is not surprising, since mycobacteria employ multiple receptors for entering host macrophages. In addition to the two receptors mentioned above, these include surfactant protein A receptors, scavenger receptors, and, in the case of opossum phagocytosis, the complement receptors CR3, CR1, and CR4 (6). Toll-like receptors are also essential for mycobacterial interactions with phagocytic cells (40), although their role in mycobacterial entry remains to be established.

Our results show that, once inside of macrophages, the replication rate and persistence of the pimF mutant appear normal. However, the interpretation of this result is not straightforward. On the one hand, it could mean that LAM does not contribute directly to the intracellular survival of the bacterium. Although LAM has been implicated to contribute to the intracellular survival of pathogenic mycobacteria by interfering with normal macrophage functions, these experiments were performed by comparing pathogenic and nonpathogenic mycobacteria or by using purified LAM (32–34). Whether the observed biological activities are caused by LAM and to what extent the observed biological functions caused by purified LAM contribute to the survival of whole bacterial cell remain to be established. On the other hand, the mutant did not exhibit apparent disadvantage in the extent of survival or rate of intracellular growth in cell culture, we cannot downplay the contribution of LAM to mycobacterial pathogenesis in vivo. LAM of pathogenic mycobacteria is considered as a virulence factor mainly through its immunosuppressive effect on host immune system (5, 11). In addition to its potential role in inhibiting phagosomal maturation, LAM is known to inhibit the production of the proinflammatory cytokine IL-12 and the subsequent production of interferon-γ by the type 1 T-cells (Th1) (5, 11). In addition, LAM binds to DC-SIGN receptor of dendritic cells, which is thought to result in inhibition of dendritic cell maturation and subsequently antigen presentation, preventing an efficient cellular immune response against M. tuberculosis infection (7, 8, 28). These effects would favor the persistence of mycobacteria in vivo (i.e. in humans or animals) but would not have been demonstrated in our current study. Future experiments to examine the pimF mutant in animal models will help to define the role of LAM in vivo. However, the current study reaffirms that the unusual mycobacterial cell wall is important to the success of the tubercle bacillus. Cell wall defects, such as that exhibited by the pimF mutant, not only affect colony morphology but also impair key steps of the infection process. As such, further analysis of morphological mutants will enhance our understanding of both cell wall biosynthesis and the role of cell wall components in mycobacterial pathogenesis.

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PimF, a Mannosyltransferase of Mycobacteria, Is Involved in the Biosynthesis of Phosphatidylinositol Mannosides and Lipoarabinomannan

David C. Alexander, Joses R. W. Jones, Tracy Tan, Jeffrey M. Chen and Jun Liu

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